Acquired Resistance to 17-Allylamino-17-Demethoxygeldanamycin (17-AAG, Tanespimycin) in Glioblastoma Cells

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Abstract

Heat shock protein 90 (HSP90) inhibitors, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin), which is currently in phase II/phase III clinical trials, are promising new anticancer agents. Here, we explored acquired resistance to HSP90 inhibitors in glioblastoma (GB), a primary brain tumor with poor prognosis. GB cells were exposed continuously to increased 17-AAG concentrations. Four 17-AAG–resistant GB cell lines were generated. High-resistance levels with resistance indices (RI = resistant line IC50/parental line IC50) of 20 to 137 were obtained rapidly (2–8 weeks). After cessation of 17-AAG exposure, RI decreased and then stabilized. Cross-resistance was found with other ansamycin benzoquinones but not with the structurally unrelated HSP90 inhibitor radicicol, the purine BIIB021, and the resorcinyl pyrazole/isoxazole amide compounds VER-49009, VER-50589, and NVP-AUY922. An inverse correlation between NAD(P)H/quinone oxidoreductase 1 (NQO1) mRNA levels and 17-AAG IC50 was observed in the resistant lines. The NQO1 inhibitor ES936 abrogated the differential effects of 17-AAG sensitivity between the parental and resistant lines. NQO1 mRNA levels and NQO1 DNA polymorphism analysis indicated different underlying mechanisms: reduced expression and selection of the inactive NQO1*2 polymorphism. Decreased NQO1 expression was also observed in a melanoma line with acquired resistance to 17-AAG. No resistance was generated with VER-50589 and NVP-AUY922. In conclusion, low NQO1 activity is a likely mechanism of acquired resistance to 17-AAG in GB, melanoma, and possibly, other tumor types. Such resistance can be overcome with novel HSP90 inhibitors. [Cancer Res 2009;69(5):1966–75]

Introduction

The molecular chaperone heat shock protein 90 (HSP90) is currently of major interest as an anticancer drug target. Through its role in regulating the conformation, stability and function of several key oncogenic client proteins, HSP90 seems to be essential in maintaining malignant transformation and in increasing the survival, growth, and invasive potential of cancer cells (1, 2). 17-Allylamino-17-demethoxygeldanamycin (17-AAG; tanespimycin) was the first-in-class HSP90 inhibitor to enter clinical trials in both adult and pediatric patients (3–5) and is currently in phase II/phase III clinical trials in adults. Signs of clinical activity have been seen with 17-AAG in different tumor types, such as melanoma, breast, and prostate cancers, and also in multiple myeloma (3, 5, 6). The combinatorial effect of HSP90 inhibitors on multiple oncogenic pathways explains the broad spectrum of anticancer activity of HSP90 inhibitors and allows them to overcome resistance to various other anticancer therapies (6–9). This combinatorial action might also render less likely the probability of cells escaping treatment with HSP90 inhibitors by using alternative resistant pathways. Known determinants of intrinsic sensitivity to geldanamycin (GA) derivatives include expression of key client proteins, e.g., ERBB2 (10), HSP90 family members (11), and cochaperone proteins, e.g., HSP72 (12), AHA1 (13), and HSP27 (14), as well as cell cycle and apoptotic regulators (15). In addition, it is known that intrinsic resistance may be due to expression of high levels of P-glycoprotein (PgP; ref. 16) and low levels of NAD(P)H/quinone oxidoreductase 1 (NQO1; DT-diaphorase; refs. 16, 17). However, there are few reports in the literature on acquired resistance to HSP90 inhibitors (18, 19). Three breast cancer cell lines with acquired resistance to GA derivatives have been described: an acquired GA-resistant cell line presenting cross-resistance with the structurally related HSP90 inhibitor herbisycin A and the cytotoxic agent doxorubicin (18) and also two acquired 17-AAG–resistant lines remaining sensitive to the structurally unrelated HSP90 inhibitor radicicol have been reported in abstract form (19). However, the mechanisms of resistance were not identified.

Glioblastoma (GB) cells are dependent on a range of activated oncoproteins and signaling pathways that require HSP90 function (20). Thus, benzoquinone ansamycin HSP90 inhibitors might be interesting agents to improve treatment results in GB, a primary brain tumor with particularly dismal prognosis (21). In support of this, all GB cell lines to date treated with GA and its derivatives, 17-AAG and the more soluble analogue 17-demethoxy-17-dimethyl-luminoethylgeldanamycin (17-DMAG; alvespimycin), were sensitive to these compounds (8, 22–24). Moreover, GA derivatives synergize with treatments used in these tumors, such as irradiation (22, 23) and anti–epidermal growth factor receptor (EGFR) therapy (24), and are able to overcome in vitro GB resistance to SN38 (8). The general concern with the development of resistance leading to cancer treatment failure may be especially relevant in GB as shown by their high rate of recurrence and their poor curability with current therapies (21). Thus, exploring possible mechanism of acquired resistance to HSP90 inhibitors in GB cells is an important

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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goal for the potential development of these drugs as part of the therapeutic arsenal against GB. In addition, such mechanisms may also be relevant to other cancers.

To understand the potential mechanisms of resistance and determine the possible impact on the clinical use of this drug, we have successfully generated in vitro acquired resistance to 17-AAG in two adult and two pediatric human GB cell lines. We show that the common acquired mechanism of 17-AAG resistance was reduced expression of NQO1. This mechanism was also identified in a BRAF mutant melanoma cell line made resistant to 17-AAG.

Materials and methods

Cell line culture. Adult (U87MG, SF268) and pediatric (KNS42) human GB cell lines were obtained from American Type Culture Collection (ATCC; LGC Promochem), National Cancer Institute (NCI), and Japan Cancer Research Resources cell bank, respectively. The pediatric line SF188 was kindly provided by Professor Daphne Haas-Kogan (University of California). The melanoma cell line WM266.4 was obtained from ATCC (16). The naturally high NQO1-expressing line HT29 was obtained from ATCC (16). The human colon cancer isogenic pair BEneg/BE2 was produced in-house (16, 25). The human colon cancer line BE vector control (BEneg) carries the NQO1*2 polymorphism, which did not alter transcription but led to an altered protein (Pro187Ser) with diminished catalytic activity and that was rapidly degraded by the ubiquitin-proteasome pathway (25, 26). Its isogenic counterpart BE-F397 clone 2 (BE2) was transfected with NQO1 (16, 25).

All lines were grown as monolayers in DMEM containing 10% FCS, 2 mmol/L glutamine, and 2 mmol/L nonessential amino acids in 5% CO2 and were free from Mycoplasma contamination (VenorGeM Mycoplasma PCR Detection kit, Minerva Biolabs).

Compounds. The ansamycin benzoquinone HSP90 inhibitors 17-AAG and 17-DMAG and their metabolite 17-amino-17-demethoxygeldanamycin (17-AG) were obtained from Axxora Ltd., Autogenbioclear, and NCI, respectively. The structurally unrelated HSP90 inhibitors used were radicicol (Sigma-Aldrich), the purine-scaffold HSP90 inhibitor BIIB021 (27), and the resorcinyl diaryl pyrazole/isoxazole amide agents VER-49009, VER-50589, and NVP-AUY922 (refs. 28, 29), which were prepared at our Institute or by Vernalis Ltd. Chemotherapeutic agents temozolomide, cisplatin, and SN38 were obtained from Apin Chemicals Ltd., Johnson Matthey Technology Center, and Sanofi-Aventis, respectively. The NQO1 inhibitor ES936 (5-methoxy-1,2-dimethyl-3-(4-nitrophenoxymethyl)[indole-4,7-dione; ref. 17), was kindly provided by Professor Christopher J. Moody (University of Nottingham). A 2 mmol/L stock solution for 17-AAG, 17-AG, and NVP-AUY922, 5 mmol/L stock solution for SN38, and 10 mmol/L stock solution for 17-DMAG, radicicol, BIIB021, VER-49009, VER-50589, and ES936 were

Figure 1. Acquired in vitro resistance to 17-AAG in GB cell lines. Resistance index (RI = IC50 ratio of resistant / parental line) for SF268-RA12 (A), U87MG-RA6 (B), SF188-RA6 (C), and KNS42-RA4 (D) resistant lines. Resistant cell lines were treated with 17-AAG continuously (drug on) or without drug pressure (drug off), as described in Materials and Methods. The IC50 values of 17-AAG in the parental and resistant cell lines are presented. Columns, mean of at least five independent experiments; bars, SD.
prepared in DMSO. Temozolomide and cisplatin were made up in saline at 20 and 2.5 mmol/L stock solutions, respectively.

Growth inhibition studies. Growth inhibition was determined using the sulforhodamine B assay (SRB), as described previously (16, 30). The IC_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control. The NQO1 inhibitor ES936 was added at the highest nontoxic concentration (10–15% of cell growth inhibition).

Development of 17-AAG acquired resistant cell lines. Early passage SF268, U87MG, SF188, and KNS42 cells were seeded into T75 flasks. The cells in one flask were serially passaged as an untreated control along with 17-AAG–treated cells in another flask at an initial concentration of 1 × IC_{50}, as previously determined by SRB. Cells were exposed continuously to compound until 80% confluent. When treated cells were able to tolerate this concentration, the compound concentration was then increased as follows: 2×, 3×, 4×, 6×, 12×, and 24× IC_{50}. The resistance index (RI) was defined by the ratio of IC_{50} resistant line/IC_{50} parental line.

Unless otherwise stated, the resistant lines were cultured without 17-AAG for at least 3 weeks before analysis.

Western blot analysis. Procedures for cell lysates and Western blotting were as previously described (31). Immunodetection was performed using antibodies listed in the supplementary data (Supplementary Table S1).

NQO1 enzyme assay. NQO1 activity was measured by a spectrophotometric assay, in which the rate of reduction of cytochrome c was monitored at 550 nm (25). Briefly, protein lysates were diluted in lysis buffer (as for Western blot) at a protein concentration of 0.5 mg/mL. An aliquot (10 μL) of the diluted protein lysate was added to the reaction mixture containing the initial electron acceptor menadione (10 μmol/L), the terminal electron acceptor cytochrome c (70 μmol/L), and NADH (500 μmol/L) as the source of reducing equivalents. All solutions were prewarmed at 37°C and assays were performed in the presence or absence of dicoumarol, an NQO1 inhibitor (1 mmol/L). NQO1 activity was taken as the dicoumarol inhibitable activity and was expressed as nanomoles of cytochrome c reduced per minute per milligram of protein. The extinction coefficient for cytochrome c of 21.1 mmol/L/cm was used in the calculations.

NQO1 mRNA levels by quantitative real-time reverse transcription–PCR. Cells were lysed in triplicate, and RNA was extracted using triazol (1 mL/T25 flask, Life Technologies Ltd.) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed using SuperScript II (Life Technologies Ltd.) and random hexamer primers (100 pmol, Invitrogen Ltd.) in a final volume of 20 μL according to the manufacturer’s instructions. RNA quantification was determined on the NanoDrop 1000 (Thermo Scientific) measuring the absorbance at 260 nm (A_{260}). RNA purity was defined by a ratio A_{260}/A_{280} of 1.9 to 2.1. NQO1 gene expression was analyzed using Assays-on-Demand Gene Expression Product Hs00168547-m1 (Applied Biosystems). TaqMan analysis was carried out according to the manufacturer’s instructions using an Applied Biosystems 7900 HT sequence detector. Each assay sample was analyzed in triplicate and multiplexed to facilitate measurement of gene expression level relative to TBP (TBP control reagents, Applied Biosystems) using the standard curve method. The PCR reactions were performed under the following conditions: 50°C for 2 min; 95°C for 5 min; 40 cycles at 95°C for 45 s, 45°C for 45 s, and 72°C for 45 s; and 1 cycle at 72°C for 10 min. Results were presented as the mean ± SD of NQO1/TBP mRNA concentration ratio.

NQO1 genotyping. The inactivating NQO1*2 polymorphism was genotyped as previously described (32). Briefly, a 284-bp fragment containing a restriction site inactivating polymorphism at the

Figure 2. Molecular signature of HSP90 inhibition by 17-AAG in SF268 parental and resistant GB lines. Western blot of cell lysates from SF268 parental line (A) and SF268-Ra6 resistant line (B) treated with 5× IC_{50} concentrations of 17-AAG of the parental line (60 nmol/L; top) and 5× IC_{50} of the resistant line (600 nmol/L; bottom). Cells were harvested at 0, 8, 16, 24, 48, and 72 h posttreatment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.
Hinf1 site was generated by PCR from genomic DNA then digested by Hinf1. The homozygous *2 genotype produced DNA fragment sizes of 152 and 132 bp, whereas the homozygous wild-type *1 remained undigested.

Glutathione S-transferase assay. Adherent SF268 parental and SF268-RA12 resistant cells were removed from T25 flasks by scraping. The cell pellet was lysed, and protein was extracted as for Western blot analysis. Three samples of each cell line at a concentration of 2.5 mg/mL of proteins were analyzed in duplicate. Total glutathione S-transferase (GST) concentration was determined with the GST assay kit from Cayman Chemical according to the manufacturer’s instructions.

Human tumor xenografts. Procedures involving animals were carried out within guidelines set out by The Institute of Cancer Research’s animal ethics committee and national guidelines (33).

Cells (2.5 × 10⁶) were injected s.c. in both flanks of female NCr athymic mice. When tumors were of 5 to 6 mm in mean diameter, mice were treated i.p. with either vehicle 43% ethanol (200 proof), 33% propylene glycol, 24% cremophor or 80 mg/kg once daily 17-AAG, five days per week. Tumors were harvested on day 11, 24 h after the last dose. Protein lysates were prepared as previously described (34) and NQO1 immunoblotting performed as above.

Statistical analysis. All values are mean ± SD of at least 3 independent experiments. Statistical significance was calculated by a two-tailed paired t test. The nonparametric two-tailed Spearman test was used to estimate the correlation between NQO1 enzyme activity and 17-AAG sensitivity. P value < 0.05 was considered statistically significant.

Results

Development of GB cell lines with acquired resistance to 17-AAG. Acquired resistance to 17-AAG developed rapidly in GB lines. SF268-RA12, SF188-RA6, and KNS42-RA4 were resistant up to 12×, 6×, and 4× IC₅₀ concentration of 17-AAG and were generated in 5, 8, and 2 weeks, respectively. There were no morphologic or growth rate differences between the resistant lines and their parental counterparts (data not shown). By contrast, the U87MG-RA6 line, which was resistant up to 6× IC₅₀ of 17-AAG, took 26 weeks to obtain and tended to grow slower than the parental line U87MG (doubling time of 57.2 ± 4.2 and 38.3 ± 9.0 hours, respectively; P = 0.0571).

The resistant lines exhibited varying levels of acquired 17-AAG resistance (Fig. 1). Under continuous drug pressure (drug on), both adult GB resistant lines presented high levels of resistance to 17-AAG with RI (RI = IC₅₀ resistant line / IC₅₀ parental line) values of 104.5 ± 11.6 (SF268-RA12) and 137.3 ± 61.6 (U87MG-RA6). RI values for the pediatric GB lines were somewhat lower: 23.0 ± 12.6 (SF188-RA6) and 20.4 ± 11.6 (KNS42-RA4). Despite its lower RI, the KNS42-RA4 sensitivity to 17-AAG (IC₅₀, 612.1 ± 280.7 nmol/L) was comparable with adult resistant lines, as the KNS42 parental line was less sensitive to 17-AAG than the other parental lines.

Increasing 17-AAG concentrations to 6×, 12×, and 24× IC₅₀ for 3 weeks in KNS42-RA4, U87MG-RA6, and SF268-RA12 did not further increase the RI values, suggesting a mechanism of resistance that was saturable. However, the RI for SF188-RA6 further increased 2.7-fold (IC₅₀, 531.2 nmol/L) when treated with 12× IC₅₀ of 17-AAG for 3 weeks.

In the absence of 17-AAG treatment (drug off), the RI decreased (3.2-fold, 3.6-fold, and 23.3-fold) in SF188-RA6, SF268-RA12, and U87MG-RA6 after 2 weeks, then remained stable up to 8.3, 26.1,
and 8.9 weeks, respectively. For KNS42-RA4, the RI increased to 33.0 ± 11.7 and then stabilized.

**Molecular effects of 17-AAG in the SF268-RA12 resistant and SF268 parental lines.** To evaluate the functional consequences of 17-AAG resistance, we compared molecular effects induced by 17-AAG treatment in the parental SF268 and the resistant SF268-RA12 lines at both 5× IC<sub>50</sub> of the parental line (60 nmol/L) and at 5× IC<sub>50</sub> of the resistant line (600 nmol/L; Fig. 2). Consistent with the molecular signature of HSP90 inhibition (3, 29), treatment of the parental line with 60 nmol/L of 17-AAG caused depletion of HSP90 client proteins ERBB2, AKT, and cyclin-dependent kinase 4 (CDK4) by 24 hours, along with an induction of HSP72. ERBB2 was the most sensitive client and was depleted as early as 8 hours; phosphorylated AKT was depleted more rapidly and to a greater extent than total AKT, consistent with previous published data (35, 36). As previously described with other client proteins (37), a transient increase in C-RAF was observed at 8 hours followed by later depletion. Although not a client protein, phosphorylation of extracellular signal-regulated kinase 1/2 was reduced as a result of depletion of upstream RAF proteins. A recovery of client protein level in the parental line was observed from 48 hours. In the resistant SF268-RA12 line treated at 5× IC<sub>50</sub> of the parental line, no depletion of CDK4 or AKT was observed. ERBB2 and CRAF were depleted at 48 hours but recovered very rapidly at 72 hours. When both the parental and resistant cells were treated at 600 nmol/L, the molecular profile of HSP90 inhibition was observed in both parental and resistant lines and no recovery of proteins was detected.

**Cross-resistance with other HSP90 inhibitors and chemotherapeutic agents.** The structurally related agents 17-DMAG and 17-AG partially circumvented acquired 17-AAG resistance. However, cross-resistance was still seen with RI values ranging between 5.1 to 7.2 and 1.5 to 12.6, respectively (Fig. 3). No cross-resistance (RI < 1.0) was found with the structurally unrelated HSP90 inhibitors, radicicol, BIIB021, VER-49009, VER-50589, or NVP-AUY922, suggesting a resistance mechanism specific to ansamycin benzoquinones.

**Figure 4.** Expression of NQO1, HSP, and DNA repair proteins in the parental and 17-AAG–resistant GB cell lines. Western blots were performed with lysates from SF268 parental and SF268-RA12 resistant lines (A), U87MG parental and U87MG-RA6 resistant lines (B), SF188 parental and SF188-RA6 resistant lines (C), and KNS42 parental and KNS42-RA4 resistant lines (D). Three samples from parental and resistant lines were harvested at 80% confluence. The resistant cells were analyzed after continuous 17-AAG pressure (+) and after cessation of 17-AAG pressure (--), as described in Materials and Methods. The human colon cancer cell line BE vector control (BE<sup>neg</sup>), which carries the inactivating NQO1*2 polymorphism, was used as the negative control (16, 25). NQO1-positive controls were the isogenic counterpart transfected with NQO1 BE-F397 clone 2 (BE2) and the naturally high-NQO1–expressing human colon cancer cell line HT29 (16). The human colon cancer cell line HT29 was used as a positive control for MGMT (47), MLH1, and MSH2 (48). The human colon cancer cell line HCT116 was used as a negative control for MLH1 (48) and as a positive control for HSP27 (49). The parental human ovarian cancer cell line CH1 and the doxorubicin-resistant cell line CH1DoxR were negative and positive controls for PgP expression (50), which was not detected in any of the GB lines (data not shown).
No cross-resistance was observed with the chemotherapeutic agents used (temozolomide, cisplatin, and SN38), except for temozolomide in SF268-RA12 (RI, 4.8 ± 1.4; \( P = 0.0167 \)).

**PgP and NQO1 expression in the parental and acquired 17-AAG–resistant GB lines.** As the intrinsic cellular sensitivity to 17-AAG is dependent on the multidrug efflux protein PgP (16) and the oxidoreductase NQO1 (16, 17), basal expression of these proteins was determined.

No detectable expression of PgP was observed in any of the parental or 17-AAG–resistant lines (data not shown).

Parental GB lines exhibited a range of NQO1 protein levels, with highest expression in SF268 cells and lowest expression in KNS42 cells (Fig. 4). Under continuous 17-AAG exposure, NQO1 protein expression was reduced to undetectable levels in the 17-AAG–resistant lines, except for SF188-RA6 wherein NQO1 protein expression was still decreased, but to a lesser extent. After cessation of 17-AAG exposure, NQO1 protein expression recovered to a level similar to their parental lines in U87MG-RA6 and SF188-RA6 cells. However, NQO1 expression remained virtually undetectable in the SF268-RA12 and KNS42-RA4 cells. These results suggest that NQO1 expression may play a role in 17-AAG–resistant GB lines.

**NQO1 enzymatic activity in GB lines with acquired 17-AAG resistance.** As expected (16, 25), there was no detectable activity in the non–NQO1-expressing BE neg colon cancer line (Table 1). NQO1 activity was 644.6 ± 299.8 nmol/min/mg of protein for the NQO1-transfected BE2 line and 8-fold higher in constitutively high-NQO1–expressing HT29 human colon cancer line. The NQO1 activity in GB parental lines was similar to the NQO1 activity in BE2. The KNS42 line expressed the lowest activity, and this was still only 2.5-fold lower than in the BE2 line.

Under 17-AAG exposure, the NQO1 activity decreased to a nearly undetectable level in the 17-AAG–resistant lines KNS42-RA4, SF268-RA12, and U87MG-RA6. In SF188-RA6, NQO1 activity decreased by 3.3-fold and remained at an intermediate level.

Cessation of 17-AAG exposure led to some recovery in NQO1 activity, except for KNS42-RA4 wherein NQO1 activity remained undetectable. SF188-RA6 NQO1 activity returned to the level of the parental line despite persistence of 17-AAG resistance. SF268-RA12 and U87MG-RA6 NQO1 levels remained intermediate.

The nonparametric two-tailed Spearman test showed an inverse correlation between the NQO1 activity and the IC_{50} of the parental and resistant cell lines, with the exception of SF188-RA6, which was an outlier (Fig. 5). These results suggest that NQO1 activity provides a potential explanation for the 17-AAG resistance in our GB lines and that an additional mechanism might also be present in SF188-RA6 line.

**Effects of NQO1 inhibitor ES936 in GB lines with acquired 17-AAG resistance.** As expected, the NQO1 inhibitor ES936 significantly reduced the cellular sensitivity to 17-AAG in the NQO1-transfected line (BE2), whereas the isogenic vector control BE neg line, which lacks NQO1 expression, was unaffected (25). We then determined the effect of ES936 on the IC_{50} for 17-AAG in the GB lines and calculated the NQO1 inhibition ratio as IC_{50} for 17-AAG in the presence of ES936/IC_{50} for 17-AAG alone (Table 2).

In the constitutively high-NQO1–expressing SF268 parental line, 17-AAG IC_{50} was significantly increased (\( P = 0.0138 \)) in the presence of ES936. In contrast, the 17-AAG IC_{50} in the non–NQO1-expressing resistant SF268-RA12 line was unaffected by ES936. This is reflected in the NQO1 inhibition ratio. Similarly, in KNS42 parental line, ES936 decreased 17-AAG sensitivity to the level of its resistant counterpart KNS42-RA4 whereas no modification was induced in this non–NQO1-expressing resistant line. In U87MG and SF188 parental lines, but to a lesser extent in their resistant

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**Table 1. NQO1 enzymatic activity and NQO1 mRNA levels in the parental and 17-AAG–resistant GB cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NQO1 activity (nmol/min/mg of protein)</th>
<th>P</th>
<th>NQO1 mRNA level (Mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>5269.1 ± 1863.4</td>
<td>NA</td>
<td>2.3 ± 1.6</td>
<td>NA</td>
</tr>
<tr>
<td>BE neg</td>
<td>&lt;2*</td>
<td>NA</td>
<td>1.5 ± 0.3</td>
<td>NA</td>
</tr>
<tr>
<td>BE2</td>
<td>644.6 ± 299.8</td>
<td>NA</td>
<td>1.3 ± 0.7</td>
<td>NA</td>
</tr>
<tr>
<td>SF268 parental</td>
<td>921.7 ± 381.8</td>
<td></td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>SF268-RA12 drug on</td>
<td>12.4 ± 14.1</td>
<td>0.0146</td>
<td>0.1 ± 0.0</td>
<td>0.0019</td>
</tr>
<tr>
<td>SF268-RA12 drug off</td>
<td>57.5 ± 14.5</td>
<td>0.0173</td>
<td>0.3 ± 0.1</td>
<td>0.0058</td>
</tr>
<tr>
<td>U87MG parental</td>
<td>600.6 ± 308.1</td>
<td></td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>U87MG-RA6 drug on</td>
<td>24.0 ± 10.7</td>
<td>0.0317</td>
<td>0.4 ± 0.2</td>
<td>0.0525</td>
</tr>
<tr>
<td>U87MG-RA6 drug off</td>
<td>169.9 ± 68.1</td>
<td>NS</td>
<td>0.5 ± 0.1</td>
<td>0.0242</td>
</tr>
<tr>
<td>SF188 parental</td>
<td>765.9 ± 229.1</td>
<td></td>
<td>1.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>SF188-RA6 drug on</td>
<td>232.8 ± 87.6</td>
<td>0.0073</td>
<td>1.3 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>SF188-RA6 drug off</td>
<td>768.8 ± 377.5</td>
<td>NS</td>
<td>1.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>KNS42 parental</td>
<td>277.8 ± 225.9</td>
<td></td>
<td>0.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>KNS42-RA4 drug on</td>
<td>4.9 ± 3.8</td>
<td>0.0490</td>
<td>0.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>KNS42-RA4 drug off</td>
<td>&lt;2*</td>
<td>0.0096</td>
<td>0.6 ± 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NOTE:** The human colon cancer cell line BE vector control (BE neg), which carries the NQO1 polymorphism leading to a proline-to-serine change in amino acid 187 with loss of NQO1 activity, was used as the negative control (16, 25). NQO1 positive controls were the isogenic counterpart NQO1-transfected BE-F397 clone 2 (BE2) and the naturally high-NQO1–containing human colon line HT29 (16). Values are mean ± SD of triplicate experiments. P values correspond to comparison of resistant versus parental line.

Abbreviations: NS, nonsignificant; NA, not applicable.

*Lower limit of detection.
counterparts, ES936 decreased 17-AAG sensitivity, reflecting the residual NQO1 activity observed in U87MG-RA6 and SF188-RA6. These results further confirm that NQO1 activity plays a major role in the mechanism of 17-AAG resistance in the GB cell lines.

**NQO1 mRNA level in GB lines with acquired 17-AAG resistance.** Quantitative real-time reverse transcription–PCR results for NQO1 are presented in Table 1. HT29 exhibited the highest NQO1 mRNA content. BEneg and BE2 had similar NQO1 mRNA levels. The NQO1 mRNA levels in the GB parental lines were similar to that in the BE lines. In the resistant line SF268-RA12, a 12-fold decrease in NQO1 mRNA level was observed under 17-AAG pressure compared with the parental line. After cessation of 17-AAG, NQO1 mRNA levels increased to an intermediate level. A similar tendency was observed with U87MG-RA6. In contrast, no change in the NQO1 mRNA levels was observed in the pediatric resistant GB lines SF188 and KNS42 with or without 17-AAG pressure. These results suggested different mechanisms of reduced NQO1 expression/activity between the adult and pediatric 17-AAG–resistant GB lines studied here.

**NQO1 genotyping in GB lines with acquired 17-AAG resistance.** As expected (16, 25), HT29 carried the wild-type NQO1*1 polymorphism (Hinf1 undigested PCR fragment), whereas BEneg/BE2 carried the inactivating NQO1*2 polymorphism (Hinf1 digested PCR fragment; Supplementary Fig. S1). Only the wild-type NQO1*1 polymorphism was detected in the parental adult GB lines U87MG and SF268 and the pediatric GB line SF188 and their 17-AAG–resistant counterparts. In the parental pediatric GB line KNS42, both NQO1*1 and NQO1*2 polymorphisms were detected whereas only the inactivating NQO1*2 polymorphism was detected in the 17-AAG–resistant line KNS42-RA4. These results suggested that the 17-AAG pressure has selected a KNS42 subpopulation homozygous for NQO1*2 polymorphism and, thus, resistant to 17-AAG.

**Reduced NQO1 expression in U87MG parental GB tumor xenografts treated with 17-AAG.** When U87MG parental adult GB tumor xenografts were treated with a therapeutic regimen of 80 mg/kg/d for 5 days a week for 2 weeks, a decrease in NQO1 protein expression was observed by immunoblot (Supplementary Fig. S2).

**Acquired 17-AAG resistance in the WM266.4 melanoma line.** To determine if the NQO1-mediated mechanism of acquired resistance to 17-AAG was restricted to GB cells, a 17-AAG–resistant melanoma line, WM266.4-RA6, was generated after 6 months of continuous drug exposure (RI, 12.5 ± 1.3). Resistance persisted but to a lesser degree after 4 weeks cessation of exposure (RI, 2.5 ± 0.3). Cross-resistance was observed with 17-DMAG but not with VER-50589 or a panel of cytotoxic drugs (Supplementary Fig. S3A). As seen in 17-AAG–resistant GB lines, NQO1 protein expression was depleted in the resistant melanoma line when compared with the parental line.

**Figure 5.** Inverse correlation between 17-AAG sensitivity and NQO1 enzyme activity in the parental and 17-AAG–resistant GB cell lines. The relationship between NQO1 activity and in vitro cellular sensitivity was evaluated in SF268 parental and SF268-RA12 resistant lines (A), U87MG parental and U87MG-RA6 resistant lines (B; SF188 parental and SF188-RA6 resistant lines (C), and KNS42 parental and KNS42-RA4 resistant lines (D). Spearman test coefficients (R) and P values are shown. The negative correlation coefficient showed an inverse relationship between NQO1 activity and 17-AAG IC50. P values of <0.05 were considered significant.
In vitro Acquired Resistance to 17-AAG

Table 2. Sensitivity to 17-AAG of the parental and 17-AAG–resistant GB cell lines and the effects of the NQO1 inhibitor ES936, as determined by SRB assay

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>17-AAG IC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</th>
<th>NQO1 inhibition ratio IC&lt;sub&gt;50&lt;/sub&gt; 17-AAG + ES936/IC&lt;sub&gt;50&lt;/sub&gt; 17-AAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17-AAG alone</td>
<td>17-AAG + ES936</td>
</tr>
<tr>
<td>SF268 parental</td>
<td>2.6 ± 1.8</td>
<td>45.5 ± 18.2</td>
</tr>
<tr>
<td>SF268-RA12</td>
<td>95.5 ± 25.6</td>
<td>100.4 ± 52.9</td>
</tr>
<tr>
<td>U87MG parental</td>
<td>4.1 ± 1.1</td>
<td>73.0 ± 42.7</td>
</tr>
<tr>
<td>U87MG-RA6</td>
<td>189.0 ± 78.8</td>
<td>346.1 ± 199.0</td>
</tr>
<tr>
<td>SF188 parental</td>
<td>4.7 ± 1.8</td>
<td>111.2 ± 43.2</td>
</tr>
<tr>
<td>SF188-RA6</td>
<td>559 ± 24.0</td>
<td>332.1 ± 9.2</td>
</tr>
<tr>
<td>KNS42 parental</td>
<td>42.1 ± 3.0</td>
<td>325.8 ± 195.8</td>
</tr>
<tr>
<td>KNS42-RA4</td>
<td>259.4 ± 116.5</td>
<td>201.5 ± 23.4</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SD of at least three independent experiments. P values correspond to comparison of ES936 treated versus control. Abbreviation: NS, nonsignificant.

parental line (Supplementary Fig. S3F). NQO1 activity (Supplementary Fig. S3C) was very high in the parental line (11.4-fold NQO1 activity in BE2), reduced in the resistant line under 17-AAG pressure to the level seen in BE2, and partially recovered after cessation of 17-AAG (3-fold NQO1 activity in BE2). NQO1 activity was inversely correlated to 17-AAG IC<sub>50</sub> (P < 0.0069; Supplementary Fig. S3C). ES936 significantly reduced WM266.4 parental line 17-AAG sensitivity to a level similar to the resistant line (P = 0.0041; Supplementary Fig. S3D) but also tended to reduce 17-AAG sensitivity in the resistant WM266.4-RA6 line, albeit to a lesser extent, due the residual NQO1 activity in this resistant line. Taken together, these results suggested that NQO1 down-regulation was likely to be the mechanism of resistance in the WM266.4-RA6 melanoma line.

Levels of HSPs in the 17-AAG–resistant GB lines. HSP90 and cochaperones HSP72 and HSP27 showed increased expression under continuous exposure to 17-AAG compared with their parental counterpart but returned to the basal level when 17-AAG exposure was stopped (Fig. 4). No HSP27 protein expression was detected in the SF268 parental/resistant lines.

Exploration of cross-resistance to temozolomide in the SF268-RA12 GB line. Figure 3 shows that the 17-AAG–resistant GB line SF268-RA12 was cross-resistant to temozolomide. ES936 did not alter the sensitivity to temozolomide in either the parental SF268 or resistant SF268-RA12 lines (data not shown), indicating the involvement of a mechanism of resistance not involving NQO1.

The absence of increased expression of mismatch repair proteins (MLH1 and MSH2 immunoblotting; Fig. 4) and increased GST activity (GST enzymatic assay; data not shown) in the resistant compared with its parental counterpart excluded the role of these mechanisms of resistance to temozolomide (38). In contrast, O<sub>6</sub>-methylguanine-DNA methyltransferase (MGMT) protein levels were undetectable in the SF268 parental line but increased in 17-AAG–resistant SF268-RA12 cell line (Fig. 4). No changes in MGMT expression were observed in the other parental/resistant pairs. The results suggest that MGMT expression might participate in the cross-resistance of the SF268-RA12 line to temozolomide.

Discussion

With the clinical development of HSP90 inhibitors and the first promising results of 17-AAG in cancer patients (3, 5, 6), it is important to understand potential mechanisms of resistance to these agents. Although some intrinsic mechanisms of resistance are known (16), few studies report acquired resistance to HSP90 inhibitors and no mechanism of acquired resistance has been described (18, 19).

This present work describes in vitro acquired resistance to 17-AAG in four human GB cell lines derived from both adult (SF268, U87MG) and pediatric (SF188, KNS42) tumors. We have shown that reduced NQO1 expression/activity is the main mechanism of resistance and extended this finding to another tumor type exemplified by a BRAF mutant melanoma cell line (WM266.4).

Cross-resistance with the analogue 17-DMAG, which is also in clinical trial, and the 17-AAG metabolite 17-AG, but not with structurally unrelated HSP90 inhibitors, led us to hypothesize mechanisms of resistance common to ansamycin benzoquinones rather than modifications to the target HSP90. We therefore explored factors previously known to have a marked effect on intrinsic sensitivity of cancer cells to 17-AAG, namely the drug efflux pump PgP (16) and the oxidoreductase NQO1 (16, 17).

PgP protein was undetectable in all the parental/resistant pairs, allowing us to exclude this mechanism. In contrast, we discovered that reduced NQO1 expression/activity was implicated in the acquired resistant phenotype of all of 17-AAG–resistant GB lines studied.

NQO1/DT-diaphorase is an obligate two-electron–reducing flavin-containing enzyme using either NADH or NADPH as reducing cofactors to catalyze the direct reduction of quinones to hydroquinones. Ansamycin benzoquinone HSP90 inhibitors, such as 17-AAG, 17-DMAG, and 17-AG, are metabolized by this enzyme to their more active hydroquinone counterpart (16, 39), and low constitutive NQO1 activity has been linked to in vitro and in vivo primary resistance to 17-AAG (16). The effect of low NQO1 activity is less for 17-DMAG and 17-AG (16, 39), thus explaining the lower degree of resistance for these two compounds in our 17-AAG–resistant lines. We observed a significant inverse correlation between NQO1 expression/activity and 17-AAG resistance (P < 0.05) and showed that the NQO1 inhibitor ES936 abrogated the differential 17-AAG sensitivity between the parental and resistant lines. In addition, analysis of NQO1 mRNA levels suggested different mechanisms leading to reduced NQO1
expression/activity in the resistant GB lines, in particular between the adult and pediatric lines studied here. In the pediatric KNS42-RA4 cells, resistance was due to the selection of a subpopulation homozygous for the inactivating NQO1*2 polymorphism, explaining the stability of the resistance after 17-AAG cessation. Interestingly, the persistence of 17-AAG resistance after cessation of treatment in the pediatric SF188-RA6 cells while NQO1 activity returned to the parental level suggested the presence of additional mechanisms of resistance, albeit restricted to GA derivatives. This will need further investigation.

Taken together, this study has clearly shown that reduced NQO1 activity, by one means or another, is a likely mechanism of in vitro acquired resistance to 17-AAG in GB. Loss of NQO1 led to high levels of acquired resistance to 17-AAG (RL, 20–137) that persisted after cessation of 17-AAG treatment (7–26 weeks), albeit at a lower RL (RL, 6–33). Resistance developed rapidly in vitro (2–8 weeks of exposure). Furthermore, reduced NQO1 expression was also observed in U87MG xenografts after only 2 weeks of 17-AAG treatment. The NQO1-mediated mechanism of 17-AAG resistance occurred in cells with high constitutive levels of NQO1 and was independent of the genetic background (different EGFR, PTEN, and p53 status; data not shown), the adult or pediatric origin of the GB cells, and the cell type (GB and melanoma). Our results suggest that a 17-AAG–induced decrease in NQO1 activity may be of potential concern for the clinical use of 17-AAG in GB, as well as in melanoma, a tumor type wherein clinical activity has been observed (3, 6). This resistance mechanism may also be applicable to other tumor types. To date, there are no published clinical data on NQO1 expression/activity in tumor biopsy samples from patients before and after 17-AAG treatment.

Several options might be considered to overcome the NQO1-mediated mechanism of resistance. Although less potent than its hydroquinone metabolite, 17-AAG itself is still an active HSP90 inhibitor (39). Our results suggest that the NQO1-mediated mechanism of resistance was saturable. Consequently, when we increased 17-AAG concentrations by a factor of 10 over a 3-day exposure in the resistant line SF268-RA12, HSP90 molecular inhibition effects were restored. However, increasing 17-AAG concentrations might have adverse effects, such as selection of additional mechanisms of resistance or increased toxicity. Hepatotoxicity was shown to be the limiting factor for 17-AAG in phase I clinical trials (3, 5, 32, 40, 41). Due to their quinone moiety, alternative ansamycin benzoquinones share the same problematic metabolism and toxic risk (39). It was not, therefore, surprising to find cross-resistance due to reduced NQO1 with both the more potent and water-soluble analogue 17-DMAG and also the main active metabolite of 17-AAG in vivo, 17-AG, thus limiting the use of these compounds as alternative therapy in 17-AAG–resistant cells.

The most appropriate strategy is probably to use structurally unrelated HSP90 inhibitors that lack quinone moiety, and several of them are currently in preclinical and clinical development (2, 5). Radicicol has not been developed further due to its lack of in vivo efficacy (42), and its derivatives are not yet in clinical trials (43). The synthetic purine-scaffold HSP90 inhibitor BIBO21 has entered phase I clinical trial. In addition, our preclinical in vitro and in vivo data suggest that the resorcinilyl diaryl pyrazole/isoxazole amides are promising HSP90 inhibitors with several advantages compared with 17-AAG, including better solubility and independence from the effects of PgP and NQO1 (29, 44). NVP-AUY922 has entered phase I clinical trial in adults (29, 45). As expected from their lack of a quinone moiety, these compounds did not exhibit any cross-resistance in our GB and melanoma 17-AAG–resistant lines. Interestingly, with the same experimental procedure used to obtain 17-AAG resistance, we have not been able to generate any resistance to VER-50589 or NVP-AUY922 over a period of exposure for up to 12 months in either GB or melanoma lines (data not shown).

As mentioned in the Introduction, it has been suggested that the combinatorial effects of HSP90 inhibition on multiple essential pathways for cancer cells might suppress activation of signaling pathways involved in drug resistance and render the probability of cells escaping HSP90 inhibition less likely (2, 45). Unfortunately, this theoretical advantage was compromised by the problematic metabolism of 17-AAG but this mechanism is not relevant to the purine and resorcinilyl diaryl pyrazole/isoxazole amide inhibitors.

Concerning the potential therapeutic significance of the loss of NQO1 expression as a possible mechanism of resistance to 17-AAG and related agents in the clinic, it would be interesting to study the effect of treatment on human GB stem cells.

In the majority of our 17-AAG–resistant lines, we did not observe any cross-resistance to cytotoxic agents, even under 17-AAG pressure. This observation was in agreement with the NQO1 mechanism of 17-AAG resistance, as none of these drugs are NQO1 substrates. The only exception was the 17-AAG–resistant SF268-RA12 line, which was also resistant to temozolomide. However, this cross-resistance seemed to be due to the selection of a subline overexpressing the DNA damage repair enzyme MGMT, which represents the most important mechanism of cellular defense against temozolomide (46).

In conclusion, low NQO1 activity is not only a mechanism of primary resistance (16) but also a likely mechanism of acquired resistance to 17-AAG in GB and melanoma. This study further highlights the problematic metabolism of GA derivatives. New series of HSP90 inhibitors that avoid the liability of NQO1 metabolism, as exemplified by the purine and resorcinilyl pyrazole/isoxazole amide analogues, are able to avoid the resistance due to decreased NQO1 activity, providing additional support for the clinical development of such structurally novel HSP90 inhibitors.

Disclosure of Potential Conflicts of Interest

N. Gaspar, S.Y. Sharp, S. Pacey, C. Jones, M. Walton, S. Eccles, A. Pearson, and P. Workman are or have been employees or students of The Institute of Cancer Research. Intellectual property from the research collaboration with Vernalis Ltd. on HSP90 inhibitors was licensed from The Institute of Cancer Research to Vernalis Ltd. and Novartis. The Institute of Cancer Research has benefited from this. The Institute of Cancer Research requires its employees to declare this potential conflict of interest. S. Eccles is consultant, Vernalis Ltd. P. Workman: commercial research grant, Vernalis Ltd.; consultant, Novartis. G. Vassal has declared no conflict of interest.

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5 www.ClinicalTrials.gov
References


Correction: Article on *In vitro* Acquired Resistance to 17-AAG

In the article on *in vitro* acquired resistance to 17-AAG in the March 1, 2009 issue of *Cancer Research* (1), there is an error in Fig. 4; the corrected figure appears below. Also, the first sentence of the Fig. 4 legend should read as follows: Expression of NQO1, HSP and DNA repair proteins in the parental and 17-AAG–resistant GB cell lines.


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**Figure 4.**
Acquired Resistance to 17-Allylamino-17-Demethoxygeldanamycin (17-AAG, Tanespimycin) in Glioblastoma Cells


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