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ORIGINAL ARTICLE

An in vitro and in vivo study of the combination of the heat shock protein inhibitor 17-allylamino-17-demethoxygeldanamycin and carboplatin in human ovarian cancer models

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Abstract
Purpose To study the interactions of the heat shock protein 90 (HSP90) inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) and carboplatin in vitro and in vivo.
Experimental design The combination of 17-AAG and carboplatin on the growth inhibition of A2780, SKOV-3, IGROV-1 and HX62 human ovarian cancer cells was studied in vitro by MTT assays. The effect of the sequence of administration of both drugs was further investigated in A2780 cells by sulforhodamine B assays. The ability of 17-AAG to deplete HSP90 client proteins either alone or in combination with carboplatin was evaluated by western blotting. Tumor concentrations of 17-AAG and carboplatin alone or in combination in vivo were determined by validated liquid chromatography with ultraviolet detection and atomic absorption spectroscopy methods. The growth inhibitory effects of 17-AAG, carboplatin and the combination were studied in the A2780 xenograft model.
Results The combination index (CI) at $fu_{0.5}$ for 17-AAG plus carboplatin was 0.97 (±0.12 SD) when A2780 cells were exposed to carboplatin followed by 17-AAG indicating additivity. The addition of carboplatin did not alter the ability of 17-AAG to cause C-RAF, CDK4 and p-AKT depletion or HSP70 induction. Tumor 17-AAG and carboplatin concentrations were not significantly different in the single agent and combination arms. Tumor weights relative to controls on day 6 (T/C) were 67% for the carboplatin, 64% for the 17-AAG and 22% for the combination.
Conclusion In the specified sequences of drug exposure, 17-AAG and carboplatin have additive growth inhibitory effects in vitro and beneficial effects were seen with the combination in vivo. These findings form the basis for the possible evaluation of 17-AAG and carboplatin in a clinical trial.

Keywords HSP90 · 17-AAG · Carboplatin · Combination studies · Ovarian cancer

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Introduction

Ovarian cancer is responsible for 3–4% of deaths from cancer in women [38]. Chemotherapy using paclitaxel, cisplatin, carboplatin and liposomal doxorubicin has important roles in the treatment of ovarian cancer in an adjuvant and metastatic setting [29]. However, the development of resistance to chemotherapy continues to be a difficult clinical problem [1]. Ovarian cancer cells are known to have abnormalities in signal transduction, such as in the RAS–RAF–MEK–ERK pathway [24, 33] and the PI3 kinase pathway [50] as well as in cell cycle control proteins [55].

HSP90 is a highly conserved intracellular molecular chaperone regulating stabilization, activation and degradation of client proteins [60]. In normal cells it plays an important role in the stress response and buffering genetic mutations at the protein level [6, 27, 31, 61]. The list of

HSP90 client proteins includes a large number that are responsible for malignant transformation and progression, including ERB-B2 [66], BCR–ABL [36], oestrogen receptors [15], androgen receptors [14], C-RAF [49], B-RAF [10, 19] CDK4 [54], AKT [46], mutant p53 [63], MET [59], h-TERT [16] and HIF1-α [34]. These oncogenic client proteins are responsible for the multiple hallmark traits of malignancy, such as deregulated signal transduction, cell cycle progression, evasion of apoptosis, immortalization, angiogenesis, invasion and metastasis [22]. Geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) compete with ATP at the nucleotide-binding site in the amino-terminal domain of HSP90 [20, 40, 53, 62]. Inhibition of the essential ATPase activity of HSP90 by these agents leads to the combinatorial degradation of multiple client proteins by the ubiquitin-proteasome pathway [48], resulting in cell cycle arrest and apoptosis [26]. These agents also inhibit invasion and angiogenesis [45]. 17-AAG is now undergoing clinical development [2, 18, 21, 37, 43, 51].

Platinum agents are active in ovarian cancer [11]. These drugs react at the N7 position of purines in DNA to form a variety of monofunctional and bifunctional adducts [25]. The mechanism of cell death due to exposure to platinum agents is believed to involve cell cycle arrest at the G2-M checkpoint, triggering apoptosis [52].

Despite the potential for single agent activity through combinatorial effects on multiple oncoproteins and signal transduction pathways [64], we reasoned that it could be useful to combine 17-AAG with platinum agents in ovarian cancer. Potential advantages of combining 17-AAG with platinum agents include the fact that the target for 17-AAG, HSP90, is involved in a variety of signaling pathways that may affect cell survival after exposure to platinum agents [57]. 17-AAG has been shown to inhibit signaling through the RAS–RAF–MEK–ERK [26] and PI3 kinase [44] pathways. Inhibition of these pathways is associated with sensitization of selected human cancer cell lines to cytotoxic drugs [39, 44]. In addition, 17-AAG and platinum agents have non-overlapping dose-limiting toxicities [2, 11], thus making them potentially attractive to use in combination in the clinic.

The combination of molecularly targeted therapies with cytotoxic agents has met with both clinical successes [4, 8] and failures [17, 23]. Robust preclinical testing of drug combinations may help predict clinical outcomes more accurately [28]. The combination of 17-AAG with cytotoxic agents such as paclitaxel, cisplatin and oxaliplatin [35, 42, 44, 57, 58], tyrosine kinase inhibitors like imatinib [41] and radiation treatment [13] has been studied. We have shown beneficial interaction between 17-AAG and paclitaxel in ovarian cancer cell lines [44]. We have also previously documented the activity of 17-AAG in A2780 human ovarian cancer xenografts [3]. We now describe the effects of combining 17-AAG and carboplatin on a panel of human ovarian cancer cells in vitro. This was followed by further experiments in the A2780 human ovarian cancer model in vitro and in vivo. The results provide support for the evaluation of this promising combination in the clinic.

Materials and methods

Drugs

17-AAG for in vitro experiments was kindly supplied by the Developmental Therapeutics Division of the National Cancer Institute (Rockville, MD, USA). 17-AAG and its vehicle (43% ethanol, 33% polypropylene glycol and 24% cremaphor) for in vivo experiments were kindly supplied by Kosan Biosciences (Hayward, CA, USA). Carboplatin and cisplatin for in vitro experiments was obtained from Sigma (Poole, UK), while carboplatin for in vivo experiments was sourced from Faulding Pharmaceuticals (Leamington Spa, UK). Unless otherwise specified, all chemicals were obtained from Sigma (Poole, UK).

Cell culture and assay

Human ovarian cancer cell lines were obtained from ATCC (Rockville, MD, USA) and The Institute of Cancer Research [44]. Cells were grown as monolayers in Dulbecco's-modified Eagle medium (Sigma, Poole, UK) containing 10% fetal calf serum (Life Technologies, Paisley, UK), 200 mM glutamine and 1x non essential amino acids (Life Technologies, Paisley, UK) in 6% CO_2.

In vitro cell growth inhibition studies

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assays: Exponentially growing A2780, SKOV3, IGROV-1 and HX62 ovarian cancer cells were plated in 96 well plates. A range of drug concentrations were added and the plates were incubated for 72 h to allow for 3–4 doubling times. MTT assays were carried out by previously validated and published techniques [44]. Each experiment was carried out in triplicate.

Sulforhodamine B (SRB) assays: Exponentially growing A2780 cells were plated in 96 well microtitre plates. For experiments studying concomitant exposure, cells were exposed to increasing concentrations of both drugs for 96 h and SRB assays were carried out by previously validated and published techniques [3]. For experiments studying the effect of sequence of exposure to 17-AAG or carboplatin,

cells were exposed to increasing concentrations of 17-AAG or carboplatin for 24 h. A period of 24-h exposure to the first agent was chosen so that the A2780 cells would be exposed to the first drug for at least one doubling time (18–24 h). The cells were then washed with sterile phosphate buffered saline (Sigma, Poole, UK) and the medium was replenished. Following this, the second drug (to which the cells were not exposed to in the first 24 h) or medium was added for 96 h. SRB assays were carried out by previously validated and published techniques [3]. All experiments were carried out in triplicate.

The results of combination studies were analyzed using the well-established principles of median effect analysis method of Chou and Talalay [7]. The effects of the combination were calculated using an in-house spreadsheet that has been validated previously [44]. For each level of fraction unaffected (fu), a combination index (CI) was calculated according to the following equation: $\mathrm{CI} = (D)_1/+(D_\mathrm{f})_1 + (D)_2/(D_\mathrm{f})_2 + \left[(D)_1(D_\mathrm{f})_2/(D_\mathrm{f})_1(D_\mathrm{f})_2\right]$, where $(D)_1$ and $(D)_2$ are the concentrations of the combination required to produce a given fu, and $(D_\mathrm{f})_1$ and $(D_\mathrm{f})_2$ are the concentrations of the individual drugs required to produce a given fu. A histogram is obtained in which CI values are given at various fu values. The combination indices in this publication are calculated for $\mathrm{fu}_{0.5}$. $\mathrm{CI} < 1$, $\mathrm{CI} = 1$ and $\mathrm{CI} > 1$ indicate synergy, additivity and antagonism respectively. A one-sample *t* test (two tailed) was used to compare the difference between the CI values at $\mathrm{fu}_{0.5}$ and unity (GraphPad prism software, Graphpad Software, San Diego, CA, USA).

Western blotting

Western blotting was carried out by previously validated techniques [3]. The primary antibodies used were C-RAF (SC-133, 1:500, Santa Cruz Biotechnology, CA, USA), heat shock protein 70 (HSP70) (SPA-810, 1:2000, Stressgen, Biotechnologies, Victoria, BC, Canada), p-AKT (4058S, 1:1,000 New England Biolabs, Hertfordshire, UK), AKT (9272, 1:1,000, New England Biolabs, Hertfordshire, UK), p-ERK 1/2 (197G2, 1:1000, New England Biolabs, Hertfordshire, UK) and GAPDH as the loading control (MAB-374, 1:5,000 Chemicon International, Temecula, CA, USA).

Tumor carboplatin levels

Tumor samples were homogenized at 1 mg/ml in phosphate-buffered saline (Sigma, Poole, UK). Quantification of carboplatin levels was determined by flameless atomic absorption spectroscopy (FAAS) using previously validated methods [5]. Analysis was carried out using an atomic absorption spectrometer (Perkin Elmer HGA-800, Beaconsfield, UK). Carboplatin standards (Faulding Pharmaceuticals, Leamington Spa, UK) of 10–150 ng/ml were prepared. Standards and samples were injected into the graphite furnace at maximum temperatures of 2,650°C and the absorbance at a wavelength of 265.9 nm was measured. The carboplatin concentration was calculated from a standard curve obtained with platinum standards. Mann–Whitney tests were carried out to compare differences in tumor drug concentrations.

Tumor 17-AAG levels

A high performance liquid chromatography method with ultraviolet detection, previously validated in our laboratory, was used [3].

Human tumor xenografts

The A2780 human ovarian cancer cell line was grown as a subcutaneous xenograft in female athymic NCr nude mice (nu/nu) by injecting 4×10^6 cells in each flank. Mice with established tumors corresponding to a mean volume of 0.69 mm^3 were randomized into groups (six animals each) for treatment with either control vehicle (43% ethanol, 33% polypropylene glycol and 24% cremaphor diluted 1:7 with sterile water) days 1–4, 17-AAG (80 mg/kg intraperitonially, days 1–4), carboplatin (60 mg/kg IP day 0) or a combination of 17-AAG (80 mg/kg IP days 1–4) and carboplatin (60 mg/kg IP day 0). Tumor growth was assessed three times weekly and tumor volumes were calculated according to a validated formula: volume $= 4.19 \times (a/4 + b/4)^3$, where *a* was the longer and *b* the shorter diameter [12]. Tumor volumes were then expressed as a proportion of the volume at the start of treatment (relative tumor volume). Thus, in the results shown, the relative tumor volume at the start of the experiment is, by definition, unity [3]. Animals were sacrificed on day 6 and tumors were dissected and weighed. A Mann–Whitney test was used to compare tumor volumes and weights between different treatment arms.

For the purpose of estimating tumor 17-AAG and carboplatin concentrations, xenografts were established on only one flank as described above. Established tumors were randomized (four animals in each group) to receive control, carboplatin 80 mg/kg IP on day 0 or 17-AAG 80 mg/kg IP (days 1–3) or a combination of both, i.e. carboplatin 80 mg/kg IP on day 0 followed 24 h later by 17-AAG 80 mg/kg IP (days 1–3). Animals were sacrificed on day 4 and tumor samples were flash frozen.

All animal procedures were approved by The Institute of Cancer Research Ethics Committee and were carried in compliance with the United Kingdom Coordinating

Committee on Cancer Research Guidelines on the Welfare of Animals in Experimental Neoplasia [65].

Results

Evaluation of the combination of 17-AAG and carboplatin in a human ovarian cancer cell line panel

Experiments were conducted to test the combination of 17-AAG and carboplatin in a panel of human ovarian cancer cell lines where cells were exposed to both drugs simultaneously in MTT assays. The combination was not synergistic in any of the cell lines and results generally indicated antagonism (Table 1). In view of the lack of synergy with simultaneous exposure, it was decided to investigate the combination of 17-AAG and carboplatin in more detail in the cell line most sensitive to 17-AAG.

Effects of scheduling on the combination of 17-AAG and carboplatin

A2780 cells were 4–14 fold more sensitive to 17-AAG than the other cell lines investigated. We therefore studied the combination of 17-AAG and carboplatin in A2780 cells. The results of median effect analysis of A2780 cells exposed to 17-AAG and carboplatin concomitantly were reconfirmed by SRB assays. The results of combination indices were similar (Tables 1, 2) and SRB assays were used for further experiments. In addition, the effect of the sequence of addition of 17-AAG and carboplatin was studied. Typical results are illustrated in Fig. 1 and the CI values at $fu_{0.5}$ from three independent experiments are summarized in Table 2. A one-sample *t* test (two tailed) was used to compare the difference between the CI values at $fu_{0.5}$ and unity. When A2780 cells were exposed to 17-AAG and carboplatin simultaneously, the CI was 2.6 ($\pm$0.21 SD, $P = 0.0058$) indicating antagonism. The CI values for the sequence 17-AAG followed by carboplatin and carboplatin followed by 17-AAG were 0.96 ($\pm$0.25 SD, $P = 0.82$) and 0.97 ($\pm$0.12 SD, $P = 0.71$) respectively, indicating additivity in both cases.

Effect of carboplatin on the ability of 17-AAG to inhibit HSP90

We have previously shown that the treatment of A2780 cells with 17-AAG provides the characteristic molecular signature of 17-AAG, comprising depletion of client proteins and induction of the co-chaperone HSP70 [3]. The effect of carboplatin on the ability of 17-AAG to inhibit HSP90 was studied by determining the expression of representative client proteins C-RAF, p-AKT and AKT, together with HSP70 in A2780 cells exposed to 17-AAG, carboplatin or the combination of 17-AAG and carboplatin at 1 $\times$ IC_{50} and 10 $\times$ IC_{50} concentrations of the respective drugs for 24 h concomitantly. In addition to the above biomarkers, p-ERK was determined as a downstream biomarker for the RAS-RAF-MEK-ERK MAP kinase pathway. The IC_{50} values of 17-AAG and carboplatin in A2780 cells determined by SRB assays were 18nM (SD $\pm$ 2.5) and 7.7 μM (SD $\pm$ 2.9), respectively.

The characteristic molecular signature of HSP90 inhibition seen when cells were exposed to 17-AAG alone was similar to that observed when cells were exposed to 17-AAG in combination with carboplatin (Fig. 2). Changes in the expression of proteins studied did not occur in cells exposed to carboplatin alone. Thus, as measured by this group of biomarkers, carboplatin did not inhibit HSP90 and carboplatin did not affect the ability of 17-AAG to inhibit HSP90.

Drug concentrations in A2780 tumor xenografts

Mice bearing established A2780 tumors were randomized into three groups (four animals each) and were treated with carboplatin 60 mg/kg IP day 0, 17-AAG 80 mg/kg days 1–3 or the combination of both drugs. Tumor drug concentrations were measured on day 4. Mann–Whitney tests

Table 1 Evaluation of the concomitant exposure of 17-AAG and carboplatin in a human ovarian cell panel

Cell line	IC_{50} 17-AAG (nM)	IC_{50} carboplatin (nM)	CI value at $fu_{0.5}$ (SD)	Significance (*P*)	Interpretation
A2780	12	6,177	2.8 (0.25)	0.007	Antagonistic
SKOV3	56	12,442	2.5 (0.46)	0.03	Antagonistic
IGROV1	70	2,233	1.5 (0.2)	0.049	Antagonistic
HX62	165	116,068	1.2 (0.15)	0.120	Additive

Median effect analysis of A2780, SKOV3, IGROV1 and HX62 cells exposed to 17-AAG and carboplatin concomitantly studied by MTT assays. The IC_{50} values for the individual drugs and CI values are shown. CI values were determined at $fu_{0.5}$. CI < 1.0 indicates synergy, CI $= 1$ additivity and CI > 1 antagonism. Significance was calculated by one sample, two-tailed *t* tests comparing values to unity

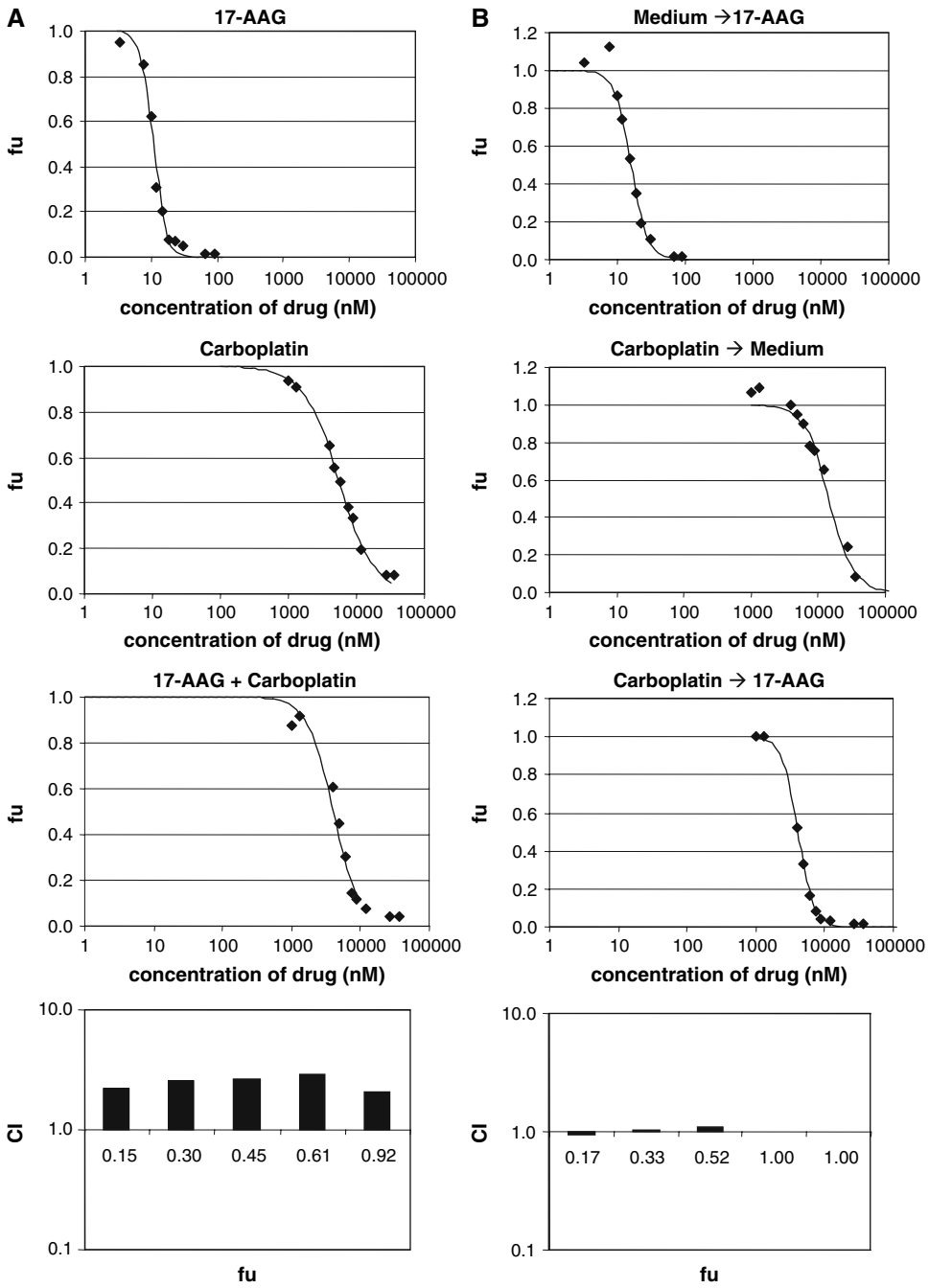

Fig. 1 Examples of median effect analysis in A2780 cells exposed to 17-AAG and carboplatin. Both drugs in the combination were added in a fixed ratio of their individual IC_{50} concentrations and the effects were analyzed using a validated [44] in-house spread sheet based on the median effect analysis of Chou and Talalay [7]. The concentration of each drug alone and the total concentrations of both drugs together are plotted against cell growth (as determined by an SRB assay) as fu values with 1.0 being the uninhibited control value. The histogram gives the CI values for different fu values using the mutually nonexclusive assumption. **a** Cells were exposed to 17-AAG and carboplatin concomitantly for 96 h and the results show antagonism. **b** Cells were exposed to carboplatin for 24 h followed by 17-AAG for 96 h and the results show additivity

Table 2 Effects of scheduling on the combination of 17-AAG and carboplatin in vitro

Sequence	CI at $fu_{0.5}$	Significance	Interpretation
17-AAG + carboplatin concomitant exposure	2.6 ± 0.21	0.0058	Antagnositic
17-AAG followed by carboplatin	0.96 ± 0.25	0.82	Additive
Carboplatin followed by 17-AAG	0.97 ± 0.12	0.71	Additive

Median effect analysis of A2780 cells exposed to 17-AAG and carboplatin in different sequences studied by SRB assays. CI values were determined at $fu_{0.5}$. CI < 1.0 indicates synergy, CI = 1 additivity and CI > 1 antagonism. Significance was calculated by one sample, two-tailed *t* tests comparing values to unity

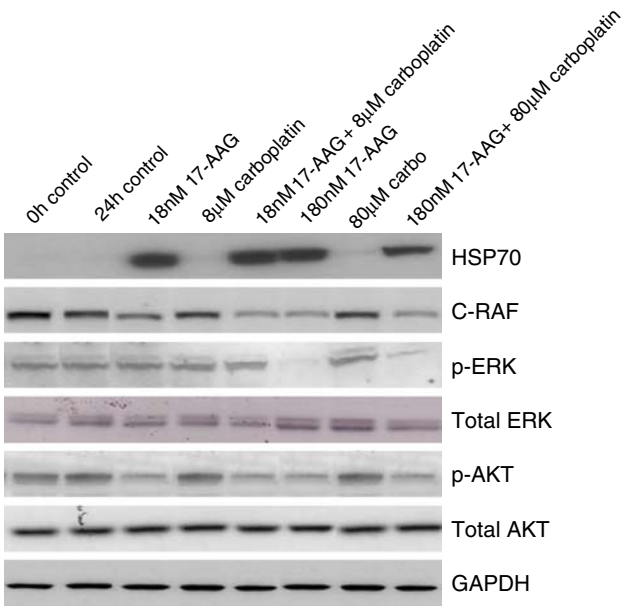

Fig. 2 Effects in A2780 human ovarian cancer cells of 17-AAG, carboplatin or the combination of both drugs on the expression HSP90 client proteins and the co-chaperone HSP70. Proteins were analyzed by western blotting with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. Cells exposed to control, 1 × IC_{50} and 10 × IC_{50} concentrations of both drugs as single agents and in combination. The IC_{50} values for 17-AAG and carboplatin were 18 nM and 8 μM respectively. Effects on client proteins C-RAF, p-AKT and AKT in addition to co-chaperone HSP70 and downstream signaling proteins p-ERK and ERK are shown. The experiment was repeated independently with similar results

were carried out to compare differences in tumor drug concentrations. The mean tumor carboplatin concentrations in the carboplatin and combination arms were similar, 93 μg/l (±30 SD) vs. 82 μg/l (±27 SD), $P = 0.63$ (Fig. 3). The 17-AAG concentrations in tumors treated with the combination of 17-AAG and carboplatin were higher than those treated with 17-AAG alone, 281 μg/l (SD 91) vs. 123 μg/l (SD 74) respectively, $P = 0.11$ although this was not statistically significant (Fig. 3).

Therapeutic effects of the combination of 17-AAG and carboplatin in A2780 tumor xenografts

Mice bearing-established tumors (mean volume 0.69 mm^3) were randomly assigned to four treatment groups with six mice in each cohort. Mice were treated with either solvent control, carboplatin 60 mg/kg IP on day 0, 17-AAG 80 mg/kg, IP on days 1–4 or a combination of carboplatin 60 mg/kg IP day 0 and 17-AAG 80 mg/kg IP on days 1–4. We previously showed that the dosing regimen of 17-AAG used resulted in decreased expression of C-RAF and CDK4 and increased expression of HSP70, confirming the inhibition of the HSP90 target in this xenograft model [3].

A2780 tumors are rapidly growing and the experiment was terminated on day 6 because of the size of the control tumors and the desire to obtain both comparative tumor volumes and relative tumor weights. Mann–Whitney tests were used to compare tumor volumes and weights between different treatment arms. Figure 4a shows the tumor growth curves for the single agent and combination treatments. Both 17-AAG and carboplatin given as single agents had a modest effect whereas the combination showed greater antitumor activity. The relative tumor volumes on day 6 for the vehicle control, 17-AAG, carboplatin and combination arms were 11.9 (±3.7 SD), 8.9 (±4.5 SD), 8.4 (±3.3 SD) and 4.2 (±2.3 SD), respectively (Fig. 4a). The day 6 tumor weights relative to control (T/C) in the 17-AAG, carboplatin and the combination arms were 64, 67 and 22%, respectively (Fig. 4b). There was a statistically significant difference between the day 6 relative tumor volume of the combination versus the 17-AAG alone arm (4.2 vs. 8.9, $P = 0.0086$) and combination versus the carboplatin alone arms (4.2 vs. 8.4, $P = 0.0024$). Further, there was a statistically significant difference between the day 6 tumor weights (*T*/*C*) in the combination versus single agent carboplatin or 17-AAG arms (Fig. 4b). The dosing regimen was well tolerated with maximum weight loss in the combination arm being 5.6%. Similar results were obtained in a repeat experiment.

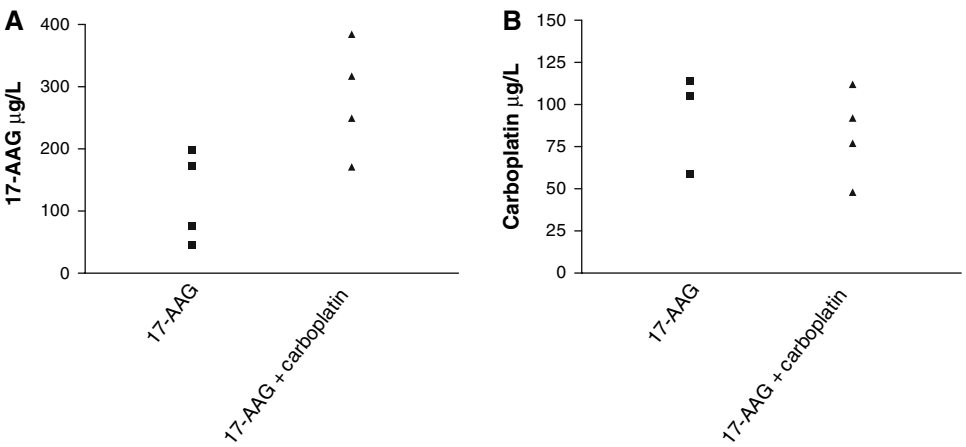

Fig. 3 Tumor concentrations of carboplatin and 17-AAG in A2780 tumor-bearing mice treated with carboplatin (60 mg/kg, IP, day 0), 17-AAG (80 mg/kg, days 1–3) or a combination of carboplatin (60 mg/kg, IP, day 0) +17-AAG (80 mg/kg, days 1–3). Tumors were analyzed for drug concentrations on day 4. **a** 17-AAG concentrations. **b** Carboplatin concentrations

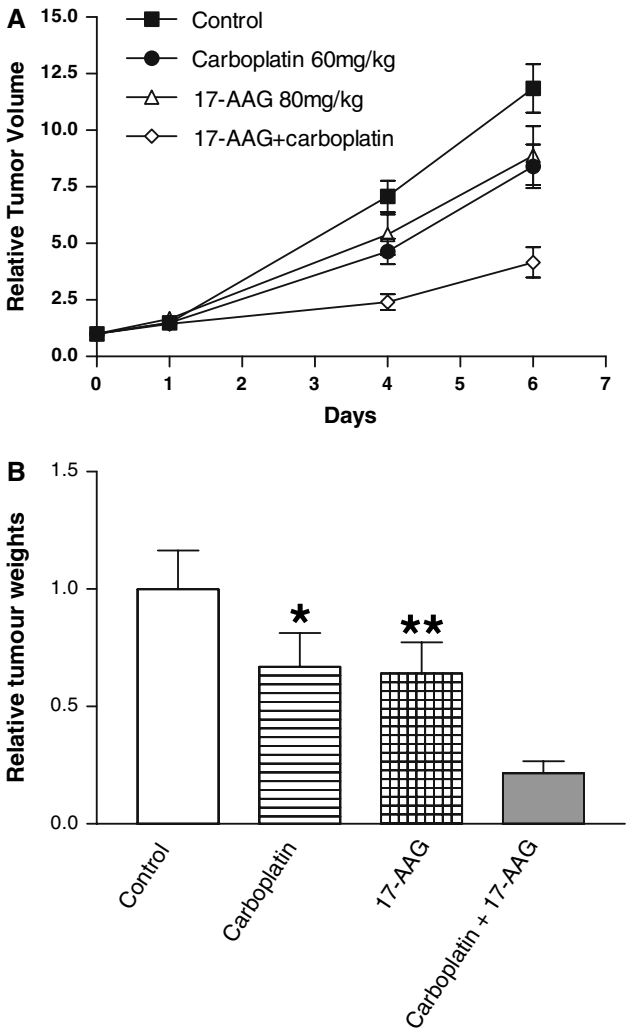

Fig. 4 The effects of 17-AAG and carboplatin, alone or in combination, on the growth of established A2780 human ovarian cancer xenografts. Mean tumor volumes at the start of treatment were 0.69 mm^3. Xenograft-bearing mice were treated with control vehicle, carboplatin (60 mg/kg, IP, day 0), 17-AAG (80 mg/kg, days 1–4) or a combination of carboplatin (60 mg/kg, IP, day 0) +17-AAG (80 mg/kg, days 1–4). **a** The relative tumor volume (normalized to initial volume on day 0) measured on days 0, 1, 4 and 6. **b** The relative tumor weights (normalized to control) on day 6. The *error bars* represent standard deviation. $*P = 0.012$ (combination vs. carboplatin), $**P = 0.015$ (combination vs. 17-AAG). Similar results were obtained in a repeat experiment

Discussion

Platinum-based chemotherapy remains an important therapeutic modality in the treatment of ovarian cancer [29]. Ovarian cancer cells have abnormalities in signal transduction pathways [24, 33, 50] and cell cycle regulation [55] and exploiting our knowledge of the molecular pathology of the disease may help improve treatment outcomes [1]. The molecular chaperone HSP90 inhibitor 17-AAG causes depletion of many oncogenic signal transduction and cell cycle proteins via the ubiquitin proteasome pathway [31].

The primary objective of the present study was to evaluate the combination of 17-AAG and carboplatin in a human ovarian cancer model. Results from a screening experiment showed no evidence of synergy when a panel of human ovarian cancer cells was exposed to 17-AAG and carboplatin concomitantly. However, A2780 cells were 4–14-fold more sensitive to 17-AAG than other cells in the panel. We thus decided to further study the combination of 17-AAG and carboplatin in this cell line. We first defined the optimal sequence of administration in the in vitro A2780 model, followed by studies to explore possible interactions at the target level. We then tested the combination of carboplatin and 17-AAG in the in vivo A2780 ovarian cancer model using the sequence of administration found to be advantageous in the in vitro experiments. We also assessed the intra-tumoral drug concentrations in the in vivo model.

When A2780 cells were exposed to 17-AAG and carboplatin concomitantly in vitro, the effect on cell growth was antagonistic. However, experiments exploring the effects of the sequence of addition of 17-AAG and carboplatin revealed that the effects on A2780 cells were additive when the cells were exposed to 17-AAG either before or after carboplatin. The reason for the antagonism seen with concomitant exposure is not clear. However, 17-AAG can cause growth arrest in the G1-S or G2-M phase of the cell cycle [26, 47], thus reducing exposure of cells in the S phase of the cell cycle to the effects of DNA damaging agents such as carboplatin. In A2780 cells, 17-AAG has a predominantly cytostatic effect and a clear G1 arrest was seen (A. Maloney, S. Sharp and P. Workman, unpublished data). Previous work on the human ovarian (SKOV-3) and colon cancer (HCT116) cell lines has shown antagonistic effects when cells were exposed to 17-AAG and platinum agents concomitantly [44, 56, 57]. However, additive effects have been observed when HCT116 cells were exposed concomitantly to 17-AAG and oxaliplatin [42]. Also, the interaction of the sequence of 17-AAG and paclitaxel in human breast (SKBR-3) and ovarian (CH1) cancer cells has been studied and optimal growth inhibition was seen when cells were exposed to paclitaxel followed by 17-AAG [35, 44, 47].

Experiments on the effects of carboplatin on the molecular signature of HSP90 inhibition [3, 44] in human ovarian cancer cells (as determined by C-RAF and p-AKT depletion together with HSP70 induction) suggested that there was unlikely to be an interaction at the level of the molecular chaperone target. The use of these biomarkers, validated in our previous pre-clinical and clinical studies [2, 3, 44], showed that the HSP90 target was inhibited by 17-AAG but not by carboplatin. This is of interest as it has

been postulated that platinum drugs may interact with the C-terminal domain of the HSP90 molecule as distinct from the interaction of 17-AAG with the N-terminal domain [32].

In vivo studies of the combination of 17-AAG and carboplatin were conducted in the A2780 xenograft model. The sequence of administration using carboplatin followed 24 h later by 17-AAG was based on findings in the in vitro experiments. 17-AAG was used in a dosing regimen for which we have previously demonstrated HSP90 inhibition [3]. There was a trend for the tumor levels of 17-AAG to be higher in the combination treatment arm, but this was not statistically significant. Relative tumor volumes and tumor weights with respect to control on day 6 in the combination arm were significantly lower compared to those in mice treated with 17-AAG or carboplatin alone. Since the doses were well tolerated, this leads us to believe that the combination is of potential clinical benefit.

Combinations of 17-AAG and cytotoxic agents are being explored clinically [37]. Preliminary data from a trial evaluating the combination of gemcitabine, cisplatin and 17-AAG showed dose-limiting toxicities of neutropenia, thrombocytopenia and elevated liver enzymes [21]. Other phase I trials exploring the combination of 17-AAG and docetaxel are still in progress [51]. Given the modest increase in therapeutic activity seen here with the combination of 17-AAG and carboplatin at well tolerated doses and the lack of overlapping clinical toxicities [2, 9, 30], our results provide support for the possible testing of the combination of 17-AAG and carboplatin in a phase I/II setting in ovarian cancer.

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