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ORIGINAL ARTICLE

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Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on Hsp90 and client proteins in melanoma models

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Abstract The heat shock protein Hsp90 is a potential target for drug discovery of novel anticancer agents. By affecting this protein, several cell signaling pathways may be simultaneously modulated. The geldanamycin analog 17AAG has been shown to inhibit Hsp90 and associated proteins. Its clinical use, however, is hampered by poor solubility and thus, difficulties in formulation. Therefore, a water-soluble derivative was desirable and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) is such a derivative. Studies were carried out in order to evaluate the activity and molecular mechanism(s) of 17DMAG in comparison with those of 17-allylamino-demethoxygeldanamycin (17AAG). 17DMAG was found to be more potent than 17AAG in a panel of 64 different patient-derived tumor explants studied in vitro in the clonogenic assay. The tumor types that responded best included mammary cancers (six of eight), head and neck cancers (two of two), sarcomas (four of four), pancreas carcinoma (two of three), colon tumors (four of eight for 17AAG and six of eight for 17DMAG), and melanoma (two of seven). Bioinformatic comparisons suggested that, while 17AAG and 17DMAG are likely to share the same mode(s) of action, there was very little similarity with standard anticancer agents. Using three permanent human melanoma cell lines with differing sensitivities to 17AAG and 17DMAG (MEXF 276L, MEXF 462NL and MEXF 514L), we found that Hsp90 protein was reduced following treatment at a concentration associated with total growth inhibition. The latter occurred in MEXF 276L cells only, which are most sensitive to both compounds. The depletion of Hsp90 was more pronounced in cells exposed to 17DMAG than in those treated with 17AAG. The reduction in Hsp90 was associated with the expression of erbB2 and erbB3 in MEXF 276L, while erbB2 and erbB3 were absent in the more resistant MEXF 462NL and MEXF 514L cells. Levels of known Hsp90 client proteins such as phosphorylated AKT followed by AKT, cyclin D1 preceding cdk4, and craf-1 declined as a result of drug treatment in all three melanoma cell lines. However, the duration of drug exposure needed to achieve these effects was variable. All cell lines showed increased expression of Hsp70 and activated cleavage of PARP. No change in PI3K expression was observed and all melanoma cells were found to harbor the activating V599E BRAF kinase mutation. The results of our in vitro studies are consistent with both 17AAG and 17DMAG acting via the same molecular mechanism, i.e. by modulating Hsp90 function. Since 17DMAG can be formulated in physiological aqueous solutions, the data reported here strongly support the development of 17DMAG as a more pharmaceutically practicable congener of 17AAG.

Keywords 17DMAG · 17AAG · Hsp90 modulation · Melanoma

Abbreviations 17AAG: 17-Allylamino-17-demethoxygeldanamycin · 17DMAG: 17-Dimethylaminoethylamino-17-demethoxygeldanamycin · 5-FU: 5-Fluorouracil · PBS: Phosphate-buffered saline · NCI: National Cancer

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Institute · DMSO: Dimethylsulfoxide · Hsp: Heat shock protein · MEXF: Melanoma xenograft established by Fiebig et al. · PARP: Poly-adenosine ribose polymerase · TGI: Total growth inhibition (no change vs initial cell number) · GI_{50}: Growth-inhibitory concentration 50% compared to control · TCA: Tumor clonogenic assay · EDTA: Ethylenediaminetetraacetic acid

Introduction

Heat shock protein 90 (Hsp90) is a prevalent protein in mammalian cells. It is an important molecule because it interacts with many different substrates whose native conformation has been changed due to environmental stresses such as heat shock. The association allows the altered proteins to regain their function. In addition it can act as a buffer for mutated proteins by correcting the inappropriate folding of the mutant form. Under normal cellular conditions, Hsp90 plays a pivotal role as molecular chaperone in the maturation of several Hsp90 client proteins. Hsp90 client proteins include various molecules that are associated with the initiation and development of cancer, such as the kinases c-raf1, erbB2, AKT (PKB) and cdk4, mutant p53, and hTERT, the catalytic subunit of the telomerase enzyme [1–4].

Since the ansamycin antibiotic geldanamycin was shown to bind to Hsp90 [5], this heat shock protein has become an attractive target for anticancer drug development. Potentially, several hallmarks of cancer could be affected simultaneously when Hsp90 function is lost, e.g. self-sufficiency in growth signals, insensitivity to antigrowth signals, limitless replicative potential and evasion of apoptosis. Geldanamycin binds to the ATP binding site of Hsp90 subsequently preventing Hsp90 activity which results in the degradation of the client proteins [5, 6]. Because of unacceptable hepatotoxicity, geldanamycin per se cannot be used clinically [7]. However, its analog 17-allylamino-17-demethoxygeldanamycin (17AAG; NSC 330507), shows reduced hepatotoxicity (see Fig. 1 for structure) whilst retaining the molecular activities of geldanamycin. It is a semi-synthetic derivative of geldanamycin [7, 8]. This compound shows a more favorable profile in terms of potency and toxicity [9]. 17AAG is currently undergoing clinical trials in several centers in the US and the UK. Its clinical use is somewhat limited, due to difficulties in formulation, mainly because of its lack of solubility in physiological fluids.

In order to provide a molecule with fewer formulation concerns, further efforts were employed by the NCI which resulted in the development of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17DMAG, NSC 707545). This molecule differs from 17AAG only in the side chain at position 17 of the ansa ring (Fig. 1). 17DMAG has considerably greater aqueous solubility than 17AAG. Investigations into the metabolism of 17DMAG have found good bioavailability with 100% and 50% obtainable after i.p. and oral delivery, respectively. Also the metabolism of 17DMAG is reduced compared to 17AAG. Furthermore, it is widely distributed to tissues [10]. However, 17AAG and 17DMAG should have similar biological profiles and mechanisms of action, i.e. modulating Hsp90. The present study was carried out in order to investigate this hypothesis. We compared the growth inhibitory effects of the two compounds, and examined the molecular consequences of treating melanoma cells with these agents. The expression of Hsp90, its clients and downstream effectors were studied. Thus, the present study is the first molecular comparison of these two structurally similar Hsp90 inhibitors.

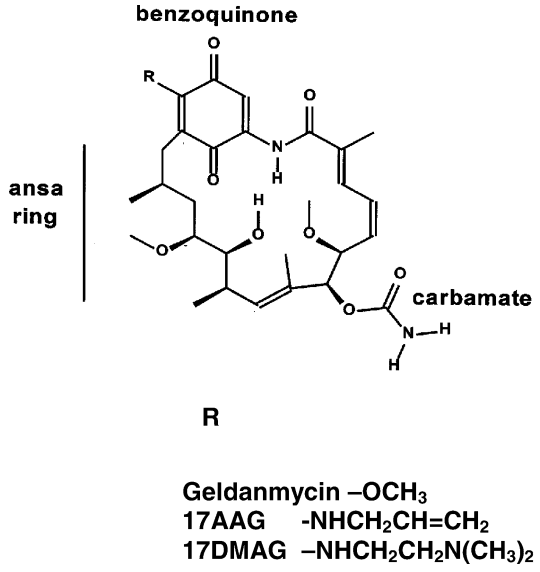


Fig. 1 Molecular structure of geldanamycin, 17AAG and 17DMAG

Materials and methods

Drugs

17DMAG (NSC 707545) and 17AAG (NSC 330507) were obtained from the Central Drug Repository of the Developmental Therapeutics Program, US NCI. In vitro studies were performed with drug prepared as a 5 m*M* stock solution in DMSO.

Cell Lines and xenografts

Human tumor xenografts were established from freshly resected tumor material or from human tumor cell lines and kept in serial passage until stable growth. The tumors were characterized for human isoenzymes and the identity with the primary was confirmed histologically [11]. Xenografts were routinely propagated a maximum of 15 passages before retrieving frozen master stocks to ensure the close relationship with the patient tumor. The

latter procedures have proven essential in our hands in order to ensure a good correlation between tumor response in patients and xenografts derived therefrom grown in nude mice or as colonies in the soft agar assay [12]. MEXF 276 and MEXF 462 are derived from lymph node and lung metastases of amelanotic melanomas, while MEXF 514 is a melanotic melanoma derived from a lymph node metastasis, which has retained its melanin production [11].

The melanoma cell lines MEXF 276L and MEXF 514L were established from the xenograft tissue (MEXF 276 and MEXF 514) as permanent cell lines in our laboratories at the University of Freiburg. MEXF 462NL was established at the NCI from MEXF 462 engraftments. Reinjected into nude mice, these cell lines resemble the histology of the original xenograft and human primary from which they were derived [13]. All experiments involving animals were performed in accordance to the German Animal Protection Act and project license regulations.

The tumor cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2 as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamicin. Cells were trypsinized upon passage and maintained routinely. All cell lines were mycoplasma free.

Tumor stem cell assay

The clonogenic assay was performed in a 24-well format according to the two-layer soft agar assay procedure introduced by Hamburger and Salmon [14] with modification. Solid human xenografts growing s.c. in serial passages in nude mice (NMRI nu/nu strain) were removed under sterile conditions, mechanically disaggregated and subsequently incubated with an enzyme cocktail consisting of collagenase (41 U/ml; Sigma, Deisenhofen, Germany), DNAse I (125 U/ml; Roche Diagnostics, Mannheim, Germany), hyaluronidase (100 U/ml; Sigma) and dispase II (1.0 U/ml; Roche Diagnostics) in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) at 37°C for 30 min. Cells were passed through sieves of 200 and 50 μm mesh size and washed twice with sterile PBS (Invitrogen). The percentage of viable cells was determined in a Neubauer hemocytometer using trypan blue exclusion. Cells (4×10^4 to 8×10^4) were added to 0.2 ml ISCOVE's medium (supplemented with 20% v/v FCS and 1% v/v gentamicin) containing 0.4% agar and plated on top of the base layer (0.75% agar). After 24 h drug was added in an additional 0.2 ml of medium. 5-FU was used as positive control. Cultures were incubated at 37°C and in a humidified atmosphere containing 7.5% CO_2 for 8–20 days and monitored closely for colony growth using an inverted microscope. During this period, in vitro tumor growth led to the formation of colonies with a diameter of >50 μm. At the time of maximum colony formation, counts were performed with an automatic image analysis system (OMNICON FAS IV, Biosys, Karben, Germany). Vital colonies were stained 24 h prior to evaluation with a sterile aqueous solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, 1 mg/ml, 100 μl/well) [15]. Drug effects are expressed in terms of percentage survival (treated/control x100, %T/C), IC_{50} and IC_{70} values.

Fig. 2 In vitro effects of 17AAG and 17DMAG (IC_{70}) in the soft agar tumor colony assay using human tumor xenografts. *Bars* represent the deviation from the mean IC_{70}. *Bars to the right* indicate more resistant, *bars to the left* more sensitive tumors in relation to the mean IC_{70} (*BXF* human bladder cancer xenograft, *CNXF* central nervous system tumor xenograft, *CXF* colon cancer xenograft, *GXF* gastric cancer xenograft, *HNXF* head and neck cancer xenograft, *LXFA* lung adenocarcinoma xenograft, *LXFE* lung epidermoid cancer xenograft, *LXFL* large-cell lung cancer xenograft, *LXFS* small-cell lung cancer xenograft, *MAXF* mammary carcinoma xenograft, *MEXF* melanoma xenograft, *OVXF* ovarian cancer xenograft, *PAXF* pancreatic tumor xenograft, *PRXF* prostate cancer xenograft, *RXF* renal cell carcinoma xenograft, *SXF* soft-tissue sarcoma xenograft, *UXF* uterine carcinoma xenograft)

Computational comparison

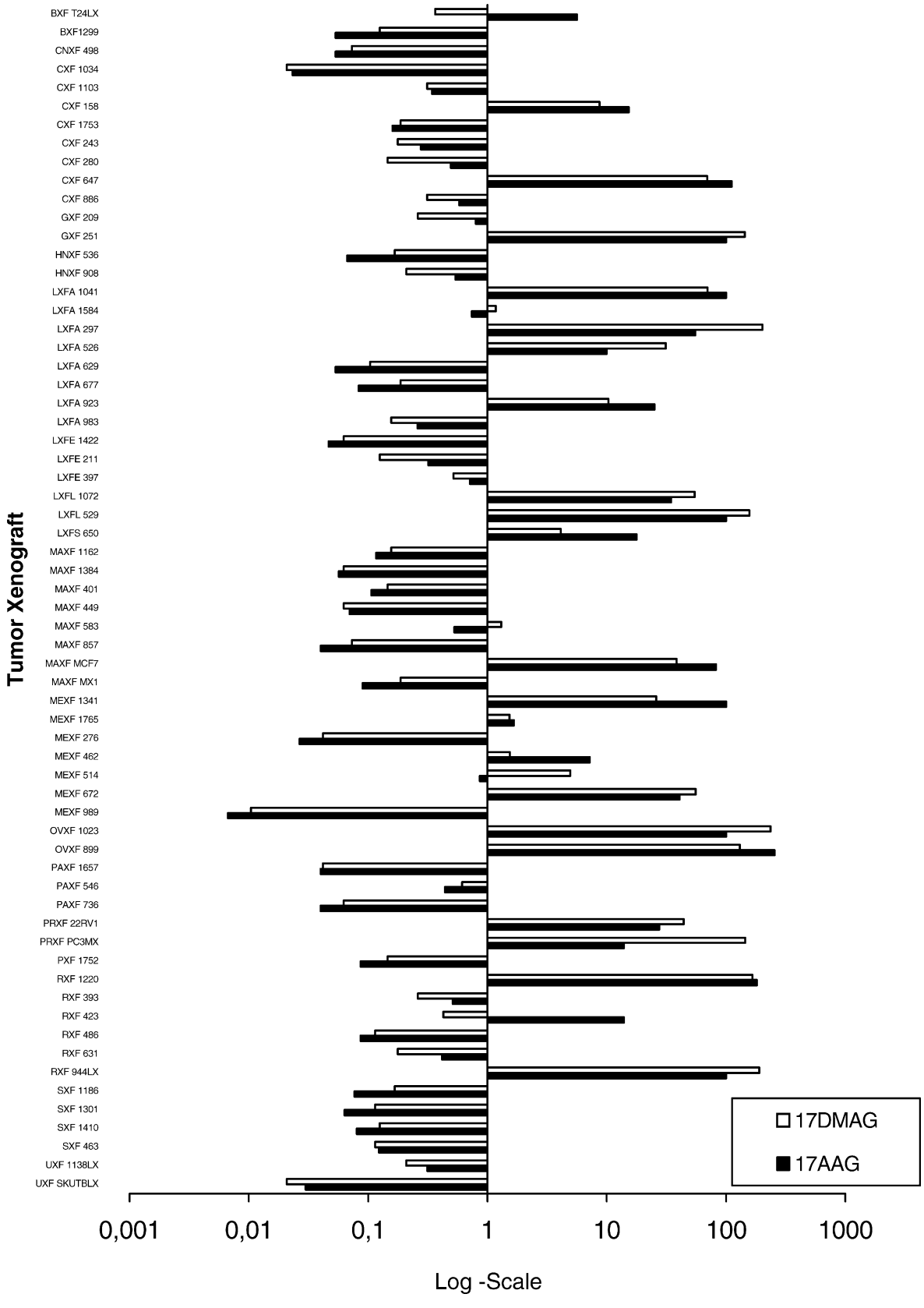
Comparisons were carried out using the data derived from the clonogenic assay. To determine the correlation between 17AAG and 17DMAG, the IC_{70} values were ranked according to Spearman's rank correlation test and the correlation coefficient rho computed [16]. The IC_{70} values of 17AAG and 17DMAG were used to compare the profile of these drugs with the IC_{70} values of clinically used anticancer agents. Spearman's rank correlation test was utilized to calculate the correlation coefficients. For both analyses, correlation values of 1 indicate matching correlation whereas values of 0 indicate no correlation.

SRB assay

The SRB assay was performed according to Skehan et al. [17]. Briefly, exponentially growing cells were harvested by trypsinization, counted and seeded into 96well plates at a concentration of 5,000 cells/well. Drug was added in 7 concentrations and total protein mass was determined after 96 hrs of continuous drug exposure by addition of sulforhodamine B (0.4%) solution. Extinctions were read at 515 nm and percentage of survival, IC_{50} and TGI values calculated.

Immunoblotting for protein expression of target and client proteins

Immunoblotting was carried out using asynchronous cells in exponential growth phase with detection by enhanced chemiluminescence. Cells (5×10^6) were seeded


Tumor Xenograft
BXF T24LX
BXF1299
CNXF 498
CXF 1034
CXF 1103
CXF 158
CXF 1753
CXF 243
CXF 280
CXF 647
CXF 886
GXF 209
GXF 251
HNXF 536
HNXF 908
LXFA 1041
LXFA 1584
LXFA 297
LXFA 526
LXFA 629
LXFA 677
LXFA 923
LXFA 983
LXFE 1422
LXFE 211
LXFE 397
LXFL 1072
LXFL 529
LXFS 650
MAXF 1162
MAXF 1384
MAXF 401
MAXF 449
MAXF 583
MAXF 857
MAXF MCF7
MAXF MX1
MEXF 1341
MEXF 1765
MEXF 276
MEXF 462
MEXF 514
MEXF 672
MEXF 989
OVXF 1023
OVXF 899
PAXF 1657
PAXF 546
PAXF 736
PRXF 22RV1
PRXF PC3MX
PXF 1752
RXF 1220
RXF 393
RXF 423
RXF 486
RXF 631
RXF 944LX
SXF 1186
SXF 1301
SXF 1410
SXF 463
UXF 1138LX
UXF SKUTBLX
17DMAG
17AAG
0,001
0,01
0,1
1
10
100
1000
Log -Scale

into tissue culture flasks and grown overnight. Cells were then exposed to either 17AAG or 17DMAG at their respective TGI concentration (MEXF 276L, 1 μ*M* 17AAG and 0.5 μ*M* 17DMAG; MEXF 462NL, 3 μ*M* 17AAG and 1.5 μ*M* 17DMAG; MEXF 514L, 10 μ*M* 17AAG and 5 μ*M* 17DMAG) for various times. At the end of the exposure time, the cells were harvested by trypsinization and lysed in a solution comprising 50 m*M* Hepes, 250 m*M* NaCl, 0.1% NP40, 10 m*M* *β*-glycerophosphate, 1 m*M* NaF, 1 m*M* EDTA, 1 m*M* Pefabloc (Roche Diagnostics), 1 m*M* DTT (Roche Diagnostics), 0.1 m*M* $NaVO_3$, 10 μg/ml aprotinin and 20 μ*M* leupeptin (all Sigma). Protein content was determined using a BioRad protein assay kit (BioRad Laboratories, Munich, Germany). Equal amounts of protein (25–50 μg) were loaded onto 4–20% Tris-glycine gradient gels (Invitrogen) and separated using SDS-PAGE. Following the transfer onto an ECL-Hybond membrane (Amersham Bioscience, Freiburg, Germany), blots were incubated with antibodies. Detection of the protein of interest was carried out by enhanced chemiluminescence (Amersham Bioscience). Densitometric analysis were performed using Kodak Digital Science 1D image analysis software, version 3.0 (Kodak, Rochester, N.Y.). The following antibodies were used: Hsp90, PI3K, c-raf1 (BD Bioscience, Heidelberg, Germany), erbB3, cyclin D1 (Labvision, Freemont, Calif.), erbB2 (DAKO, Hamburg, Germany), Hsp70 (Stressgen, Biomol, Hamburg, Germany), PARP, Akt, phospho-Akt (ser 473), cdk4 (New England Biolabs, Frankfurt, Germany), and actin (as loading control; Oncogene, Boston, Mass.). Secondary antibodies were purchased from Amersham Bioscience.

Analysis of BRAF mutations

MEXF 276L, MEXF 514L and MEXF 462NL melanoma cell lines were examined for BRAF mutations (V599E) in exon 15. The V599E *mt* BRAF colon cancer cell line HT29 was used as a positive control, and the *wt* BRAF breast cancer cell line MDA-MB-231 as a negative control. Genomic DNA was isolated from exponentially growing cells in culture using a QIAmp DNA Mini kit (Qiagen, Hilden, Germany). DNA (345 ng) was amplified using Platinum Taq (Invitrogen, Carlsbad, Calif.) polymerase with exon 15 primers (BRAF15, f-TCATAATGCTTGCTCTGATAGGA; BRAF15, r-GGCCAAAAATTTAATCAGTGGA) under conditions described previously [18]. PCR products were separated on a 1.2% agarose gel, excised and cleaned with a gel purification kit (Qiagen). The purified BRAF product (4 ng) of about 230 bp was sequenced using a Big Dye version 3.0 kit with 0.8 p *M* forward exon 15 primer, POP4 polymer and an ABI PRISM 3100 V 3.7 automated sequencer (ABI, Foster City, Calif.). Data were analyzed with ABI SeqScape v 2.0 software.

Results

Tumor stem cell assay

In the NCI 60 cell line panel the mean GI_{50} level for 17AAG was 123 n *M*, whereas it was 53 n *M* for 17DMAG (data not shown). Tumor stem cell growth was evaluated in the clonogenic assay. In the present study the antiproliferative activities of 17AAG and 17DMAG were investigated in 64 different tumors representing various tumor types. The mean IC_{70} values of 17AAG and 17DMAG were 300 n *M* for and 96 n *M*, respectively. Thus, 17DMAG was threefold more potent. Comparing the overall response profile to the two agents, the graphs can be superimposed (Fig. 2). The tumor types that responded best include breast cancers (six of eight), head and neck cancers (two of two), sarcomas (four of four), pancreas carcinomas (two of three), colon tumors (four of eight for 17AAG, six of eight for 17DMAG), and melanomas (two of seven). The melanoma panel showed a variety of sensitivities within the same tumor type. As reported previously, the MEXF 276 and MEXF 989 tumors were the two most responsive models amongst the melanomas [19]. Moreover, MEXF 276 was the most responsive tumor of the whole panel investigated. MEXF 514 and MEXF 462 were consistently found to be less sensitive. From three of the melanomas, stable cell lines have been established in our laboratories, namely MEXF 276L (derived from MEXF 276), MEXF 514L (from MEXF 514) and MEXF 462NL (from MEXF 462). Using these lines, we were able to examine the growth inhibitory effects of the Hsp90 modulators as well as their molecular mechanism(s) of action in cells with different sensitivities to these agents but derived from the same histology.

Monolayer assay

The effects of the test compounds on all dividing cells were evaluated in a monolayer assay. The results of the SRB proliferation assay confirmed the results of the clonogenic assay (Table 1), in that MEXF 276L cells were the most sensitive cells of the three investigated: the IC_{70} values of 17AAG and 17DMAG were 190 n *M* and 60 n *M*, respectively. MEXF 462NL and MEXF 514L were less sensitive to both compounds. Furthermore, 17DMAG was more potent than 17AAG in all three melanoma cell lines tested (Table 1).

Computational comparison of 17AAG and 17DMAG in the clonogenic assay

The computational algorithm COMPARE utilizes the correlation between the growth-inhibitory pattern of agents to explore relatedness between mechanisms [20].

Table 1 Mean growth-inhibitory effects (IC_{50}, IC_{70}, IC_{90}) of 17AAG and 17DMAG determined in three melanoma cell lines in vitro by the SRB assay. The data presented are the means ± SE (μM) from at least three independent experiments

	17AAG	17DMAG
IC_{50} (μM)		
MEXF 276L	0.05 ± 0.02	0.04 ± 0.01
MEXF 462NL	0.48 ± 0.09	0.09 ± 0.00
MEXF 514L	38.33 ± 4.41	7.65 ± 4.17
IC_{70} (μM)		
MEXF 276L	0.19 ± 0.06	0.06 ± 0.03
MEXF 462NL	0.83 ± 0.07	0.16 ± 0.00
MEXF 514L	61.25 ± 8.75	8.00 ± 1.53
IC_{90}(μM)		
MEXF 276L	4.38 ± 2.14	1.07 ± 0.48
MEXF 462NL	2.60 ± 0.71	0.93 ± 0.05
MEXF 514L	127.50 ± 13.15	58.33 ± 10.93

We utilized an analogous approach with Spearman's rank correlation for comparing the IC_{70} values obtained for 17AAG and 17DMAG, and found a coefficient of correlation (rho) value of 0.95 [16]. No correlations between 17AAG or 17DMAG and several commonly used anticancer agents were identified (Table 2). Therefore 17AAG and 17DMAG must affect cells via a mechanism different from those utilized by any of these known compounds, suggesting a unique mechanism of action. These findings together with the superimposable antiproliferative profile of 17AAG and 17DMAG suggest that the two compounds act by the same mechanism. In order to verify this, the effects of both agents on their putative target, namely Hsp90, as well as a number of client proteins of Hsp90 were investigated.

Table 2 Correlation of 17AAG and 17DMAG with standard anticancer agents. The data presented are the correlation coefficients (rho), where 1 indicates perfect correlation, 0 no correlation, and −1 perfect inverse correlation

Drug	No. of comparisons	Correlation coefficient (rho)	
		17AAG	17DMAG
17AAG	21	1.0	0.95
17DMAG	21	0.95	1.0
4-Hydroxy-ifosfamide	17	0.38	0.33
Bleomycin	19	0.37	0.28
Vincristine	19	0.25	0.19
Adriamycin	21	0.14	0.23
Etoposide	21	0.14	0.28
Mitoxantrone	21	0.09	0.14
1-(2-Chloroethyl)-1-nitroso-3-(2-hydroxyethyl)-urea (HECNU)	20	0.08	0.13
5-Fluorouracil	21	0.08	0.11
4-Hydroxy-cyclophosphamide	20	0.05	0.26
Vinblastine	15	0.00	−0.08
Vindesine	20	0.00	0.04
Cisplatin	21	−0.17	−0.07
Dacarbazine citrate	18	−0.07	−0.02

Molecular mode of action

To assess the effects of 17AAG and 17DMAG on their target and its client proteins, melanoma cell lines were incubated for various lengths of time with 17AAG and 17DMAG at their respective TGI, i.e. pharmacologically equitoxic, concentrations. Analysis of the drug target Hsp90 showed a decrease in protein expression in MEXF 276L cells, but not in MEXF 462NL or MEXF 514L cells. This decrease was more pronounced in cells treated with 17DMAG than in those treated with 17AAG. Several client proteins of Hsp90 are known. Some of these were investigated in cells after exposure to 17AAG and 17DMAG (Fig. 3, Table 3).

The most striking difference between the most sensitive cell line MEXF 276L, the intermediate cell line MEXF 462NL and the least sensitive cell line MEXF 514L was the expression of erbB2. ErbB2 has been previously proposed as an important client protein and effector of Hsp90 inhibition [21–23]. ErbB2 was expressed in MEXF 276L cells (Fig 3d), but not in the other two cell lines. When MEXF 276L cells were exposed to either Hsp90 modulator, a reduction in erbB2 protein expression starting 4–8 h after initiation of treatment was observed. Another member of the family of epidermal growth factor receptors, erbB3, was also expressed in MEXF 276L cells only. Expression of this protein, however, did not decline until at least 48 h of treatment with 17AAG, and remained unchanged with exposure to 17DMAG (Fig. 3a).

In the geldanamycin-sensitive cell line MEXF 276L, the expression of Hsp90 and most of its client proteins (c-raf1, pAKT, AKT, cyclin D1, cdk4)—with the exception of PI3K—decreased, while cleaved PARP and Hsp70 levels increased (Fig. 3a, Table 3). MEXF 462NL cells exhibited essentially the same pattern of kinetics of Hsp90 client protein expression as MEXF 276L cells, PARP cleavage and Hsp70 were also induced (Fig. 3b, Table 3). In contrast, in 17AAG/17DMAG-resistant MEXF 514L cells, craf-1 levels remained unchanged and pAKT and AKT expression decreased much later, with inhibition seen only at 48 and 96 h, respectively (Fig. 3c, Table 3). Moreover, Hsp70 induction was less pronounced and PARP cleavage was more delayed (Fig. 3c). In MEXF 462NL and MEXF 276L cells, c-raf1 protein expression was reduced after 16 and 24 h of drug treatment, respectively.

AKT was downregulated following an early drop in pAKT levels in MEXF 276L and MEXF 462NL cells, but more so in the drug-sensitive MEXF 276L line (Fig. 3). These findings are consistent with those of previous studies showing a rapid fall of Akt phosphorylation prior to any decline in Akt protein expression in breast cancer cells [23].

Cdk4 is another important client protein of Hsp90. Together with cyclin D it forms a kinase complex that is crucially involved in the regulation of transition of cells through G_1 to S phase of the cell cycle. Cdk4 expression was decreased in all cell lines starting after 4 h (MEXF

462NL and MEXF 514L) and 16 h (MEXF 276L) of treatment. Consequently cyclin D1 protein levels declined from 24 h onwards after exposure to either inhibitor.

Several studies have shown Hsp70 protein levels to increase when Hsp90 is inhibited [24–26]. The present study confirmed these observations. An increase in Hsp70 protein expression was observed following 4–8 h of exposure to 17AAG or 17DMAG. The elevation in Hsp70 was found in all three cell lines investigated irrespective of their sensitivity to 17AAG or 17DMAG. Furthermore, densitometric analysis revealed that the induction of Hsp70 was greater after exposure to 17DMAG than after exposure to 17AAG (two- to threefold for 17AAG vs four- to sixfold for 17DMAG).

Whether 17DMAG causes apoptosis was studied by examining the protein expression of PARP. The cleaved form of PARP (85 kDa) was observed within 16 h of treatment with either 17AAG or 17DMAG at their respective TGI concentrations in the more sensitive MEXF 267L and MEXF 462NL cells, but appeared only between 48 and 96 h in the less sensitive MEXF 514L cells. This is in accordance with the findings of previous studies of 17AAG [2, 21], and is evidence that 17DMAG at its TGI concentration can also activate apoptotic cell death pathways.

Relationship between drug response and mutant BRAF status in melanoma

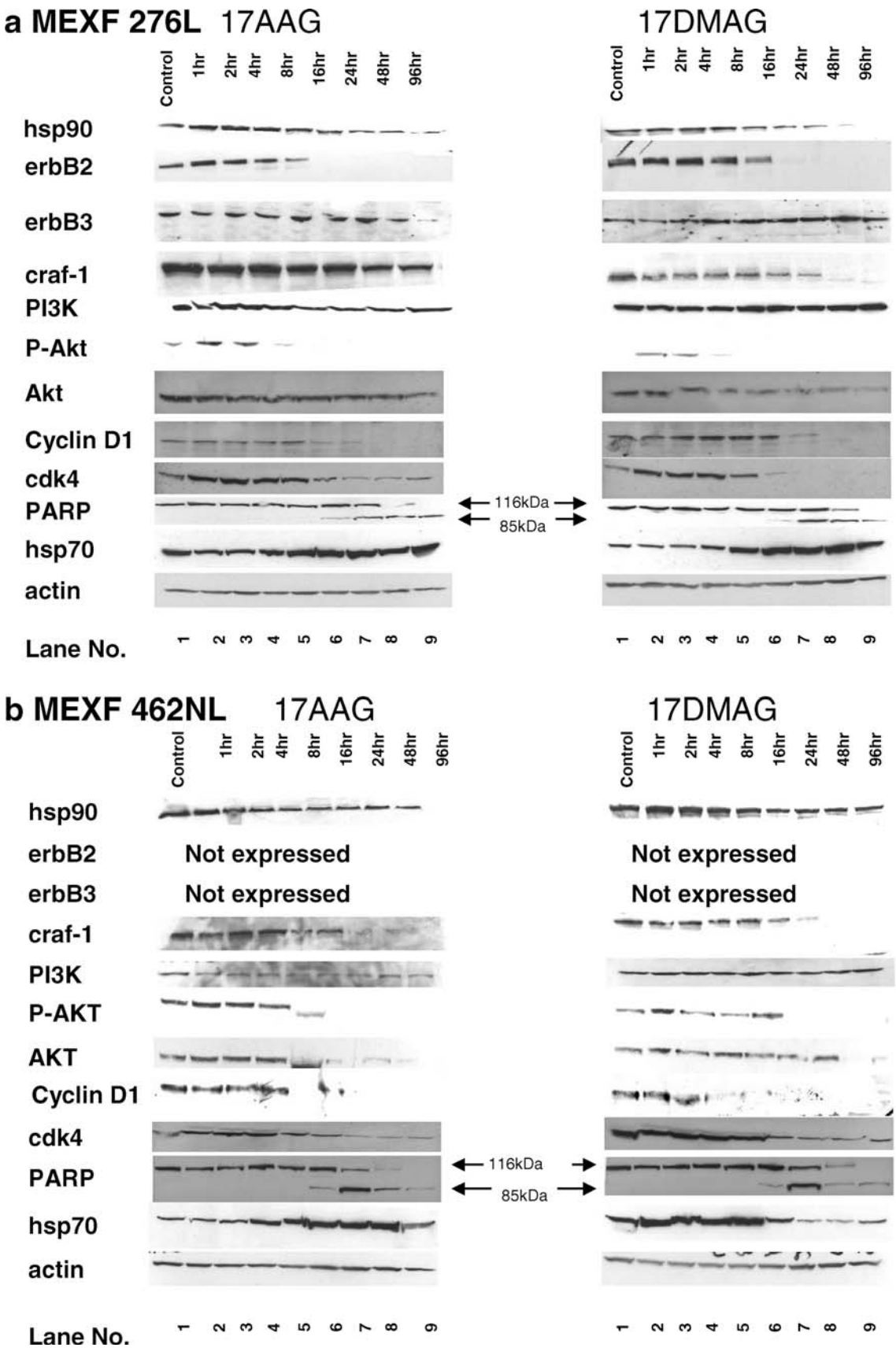
To evaluate whether the sensitivity of melanoma cell lines is related to the presence or absence of an activating BRAF mutation in the kinase domain, the three malignant melanoma cell lines used to examine the effects of 17DMAG and 17AAG on Hsp90 client proteins were analyzed for a V599E mutation. This mutation is a single base pair substitution in the activation segment of the kinase domain and accounts for 80% of all BRAF mutations found in malignant melanoma. The mutation leads to elevated kinase activity [18, 27]. All melanoma cell lines used here did exhibit the BRAF V599E mutation in exon 15 (Fig. 4). This point mutation is characterized by the replacement of a thymine (T) by an adenine (A) base at nucleotide 1796 of the genomic BRAF DNA sequence which results in a valine (V) to glutamic acid (E) amino acid change [18, 27] (Fig. 4). However, sensitivity of melanoma cell lines to 17AAG and/or 17DMAG appears not to be related to BRAF status.

Discussion

17AAG is an anticancer agent developed by the US NCI as part of a target-based program directed at the molecular chaperone Hsp90. It retains activity in xenograft models, but has much less liver toxicity than the parent molecule geldanamycin. The difficulties encountered with the clinical formulation of 17AAG led to further efforts to design a water-soluble analog and resulted in the development of the derivative 17DMAG (NSC 707545). The aim of the present study was to compare in vitro activity and mechanism(s) of action of 17AAG and the derivative 17DMAG. Both agents showed marked in vitro antitumor activity with 17DMAG being more potent than 17AAG. The mean IC_{70} of 17DMAG over 64 different tumors was two- to threefold less than that of 17AAG (mean 96 n *M* and 300 n *M*, respectively). Several tumor types showed pronounced sensitivity: breast cancer, head and neck cancer, sarcoma, pancreas carcinoma, colon tumors and melanoma. These tumors are good candidates for in vivo investigations and potential target tumor types for phase II clinical trials.

Previous studies in our laboratory employing melanomas as a test model have shown pronounced in vivo activity of 17AAG in melanoma xenografts. Moreover, in phase I clinical trials in the UK and the US responses have been seen in melanoma patients [19, 28, 29]. Therefore, and because melanoma lines exist that covered a broad range of sensitivities, this tumor type was selected for molecular mechanism studies in vitro. The three melanoma cell lines MEXF 276L, MEXF 462NL and MEXF 514L showed the same ranking in the SRB assay and the clonogenic assay: MEXF 276L cells were the most sensitive and MEXF 514L cells were the least sensitive of the three cell lines. Overlaying the 17AAG/17DMAG in vitro response profiles, the graphs showing the IC_{70} values could be superimposed (Fig. 2). This strongly suggests that the two compounds act by similar mechanisms. A lack of correlation with a variety of commonly used anticancer agents indicates that the modes of action of 17AAG and 17DMAG differ from those of currently used chemotherapeutics. These hypotheses were confirmed by investigating the effects of

Fig. 3 **a** Western blot analysis of various target and client proteins of Hsp90 in MEXF 276L cells following treatment with 17AAG or 17DMAG at their respective TGI concentrations (1 μ*M* for 17AAG and 0.5 μ*M* for 17DMAG). A decline in protein expression of the target Hsp90 and the client proteins erbB2, craf-1, phospho-AKT, AKT, cyclin D1 and cdk4, and induction of PARP cleavage and Hsp70 expression are shown. **b** Western blot analysis of various target and client proteins of Hsp90 in MEXF 462NL cells following treatment with 17AAG or 17DMAG at their respective TGI concentrations (3 μ*M* for 17AAG and 1.5 μ*M* for 17DMAG). A decline in protein expression of the client proteins craf-1, phospho-AKT, AKT, cyclin D1 and cdk4, and induction of PARP cleavage and Hsp70 expression are shown; Hsp90 protein levels are not changed. **c** Western blot analysis of various target and client proteins of Hsp90 in MEXF 514L cells following treatment with 17AAG or 17DMAG at their respective TGI concentrations (10 μ*M* for 17AAG and 5 μ*M* for 17DMAG). A decline in protein expression of the client proteins cyclin D1 and cdk4, and induction of PARP cleavage and Hsp70 expression are shown; Hsp90, craf-1 and PI3K expression did not change. **d** Western blot analysis of expression of erbB2 and erbB3 in MEXF 276L, MEXF 462NL and MEXF 514L cells. Detectable levels of these proteins are only seen in MEXF 276L cells


a MEXF 276L 17AAG
17DMAG
Control
1hr
2hr
4hr
8hr
16hr
24hr
48hr
96hr
hsp90
erbB2
erbB3
craf-1
PI3K
P-Akt
Akt
Cyclin D1
cdk4
PARP
hsp70
actin
116kDa
85kDa
Lane No.
1 2 3 4 5 6 7 8 9
b MEXF 462NL 17AAG
17DMAG
Not expressed
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P-AKT
AKT

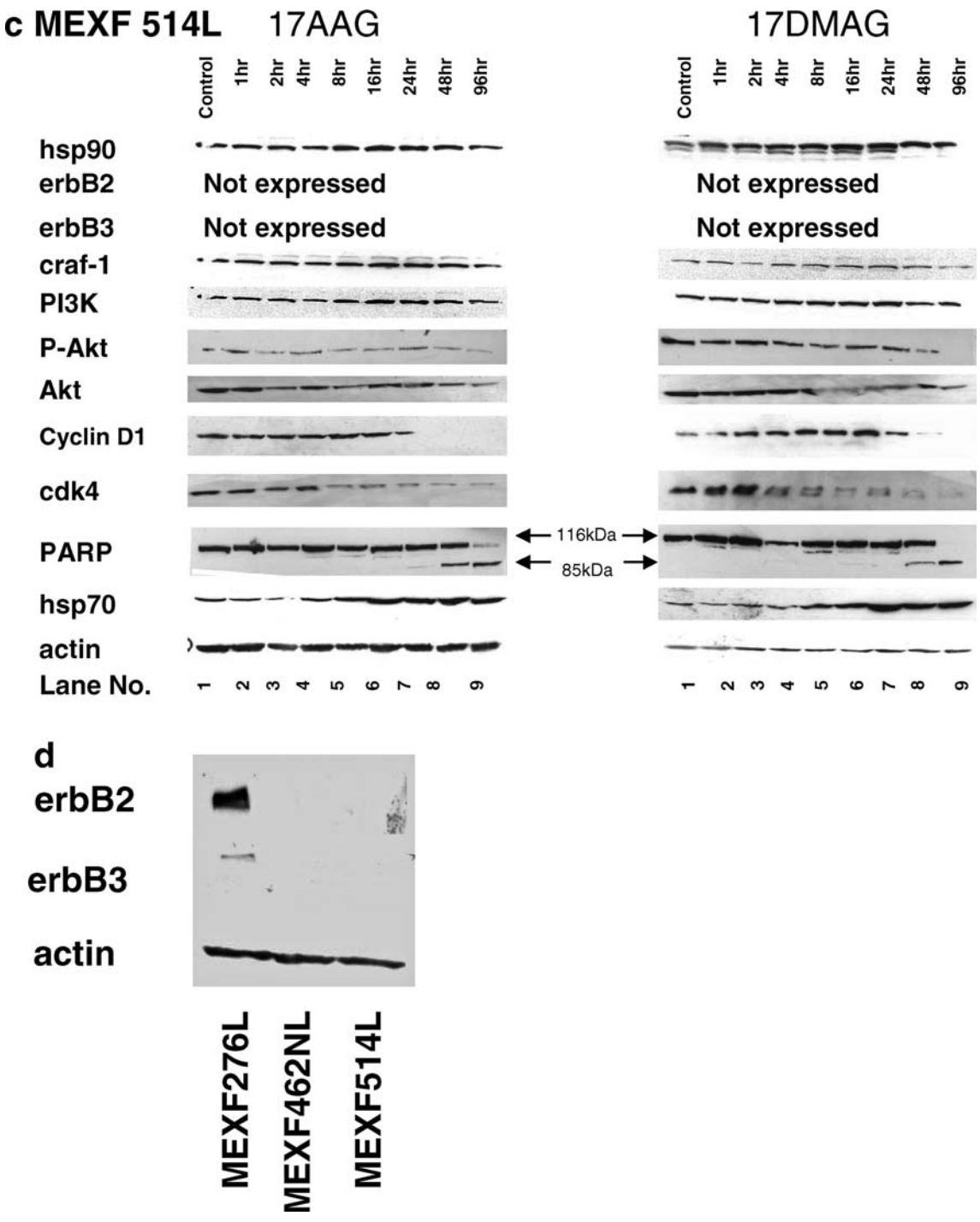

Fig. 3 (Contd.)

the two agents on a number of proteins that have been associated with Hsp90 modulation [1–4].

In the most sensitive cells (MEXF 276L) the expression of the target protein Hsp90 was decreased upon treatment with either 17AAG or 17DMAG. In contrast, no effect on Hsp90 was observed in cells less sensitive to these compounds. Nonetheless, all cell lines investigated showed a marked increase in Hsp70 protein expression which is often found in response to stress and has been associated with inhibition of Hsp90 activity [24, 26]. These findings, together with those of previous studies, are in accord with Hsp90 being the target of 17AAG, and suggest that 17DMAG also targets Hsp90. The mode of depletion of Hsp90 by 17AAG and 17DMAG in MEXF 276L cells is currently under investigation in our laboratories. It is, however, likely that reduction of Hsp90 expression is related to an enhanced ubiquitinylation of Hsp90 when bound to drug and thus its accelerated degradation in the ubiquitin-proteasome system (UPS).

Table 3 Changes in protein expression as determined by immunoblotting following treatment with 17AAG or 17DMAG at their respective TGI concentrations in the cell lines MEXF 276L, MEXF 462NL, and MEXF 514L (↓ reduction in protein expression, ↑ increase in protein expression, *NC* no change, *NEx* not expressed)

Compound	Hsp90	Hsp90 client protein									
		ErbB2	Craf-1	PI3K	P-Akt	Akt	Cyclin D1	Cdk4	Cleaved PARP	ErbB3	Hsp70
MEXF 276L											
17AAG	↓	↓	↓	NC	↓	↓	↓	↓	↓	↓	↓
17DMAG	↓	↓	↓	NC	↓	↓	↓	↓	↓	NC	↓
MEXF 462NL											
17AAG	NC	NEx	↓	NC	↓	↓	↓	↓	↓	NEx	↓
17DMAG	NC	NEx	↓	NC	↓	↓	↓	↓	↓	NEx	↓
MEXF 514L											
17AAG	NC	NEx	NC	NC	↓	↓	↓	↓	↓	NEx	↓
17DMAG	NC	NEx	NC	NC	↓	↓	↓	↓	↓	NEx	↓

A recent study has shown that the chaperone-dependent ubiquitin E3 ligase CHIP associates with erbB2. Most importantly the ligase is recruited to the erbB2/Hsp90 complex by geldanamycin and induces ubiquitinylation of erbB2 [30]. MEXF 276L cells are distinct from the more resistant melanoma cell lines through expression of erbB2. Interestingly, gene expression data derived from analysis of the Freiburg panel of human tumor xenografts and cell lines on HG-U95Av2 Affymetrix arrays have revealed that mRNA levels for the CHIP ubiquitin ligase are about fivefold higher in MEXF 276 tumor cells (normalized mean signal intensity 1170) than in resistant MEXF 514L cells (normalized mean signal intensity 249) [12]. The fact that erbB2 binds to Hsp90 and that this complex in concert with geldanamycin analogs attracts CHIP and initiates degradative processes, support the hypothesis that decreases in Hsp90 levels in the 17AAG/17DMAG-sensitive cell lines are associated with its degradation in the UPS rather than changes at the transcriptional level. This is further underlined by the finding that interaction of Hsp90 with drugs such as hypericin leads to its diminution via ubiquitination and not Hsp90 mRNA levels [31].

The importance of the expression of erbB2 for the enhanced sensitivity of cancer cells to 17AAG has been frequently reported [21–23, 30, 32]. Breast and ovarian cancer cell lines transfected with the gene encoding erbB2 are more sensitive to 17AAG treatment than nontransfected cells [21, 32]. In breast cancer cell lines, 17AAG depletes erbB2 protein expression as well as cyclin D1 and it has been shown that the reduction in erbB2 expression is necessary but not sufficient for growth arrest and apoptosis. For the latter, inhibition of signaling through an erbB3-PI3K-AKT-mediated pathway seems to be required [21]. AKT activation in breast cancer cells expressing high erbB2 levels is dependent on erbB2/erbB3 heterodimer formation and is necessary for D-cyclin expression. 17AAG has been shown to target this pathway via inhibition of erbB2 and AKT activity [23]. It has been further observed that the absence of erbB3 and constitutive activation of PI3K may actually confer resistance to 17-AAG [21].

The most significant difference between the melanoma lines showing varying sensitivities to 17AAG and 17DMAG investigated in the present study was in their level of expression of erbB2 protein. High sensitivity to 17AAG or 17DMAG and expression of erbB2 and erbB3 were associated, supporting previous findings in breast and medulloblastoma cells [21–23]. In line with 17AAG activity in erbB2-positive breast cancer cells, treatment of MEXF 276L cells (the only cell line in the panel to express erbB2) with either of the two Hsp90 modulators, resulted in degradation of erbB2 and con-

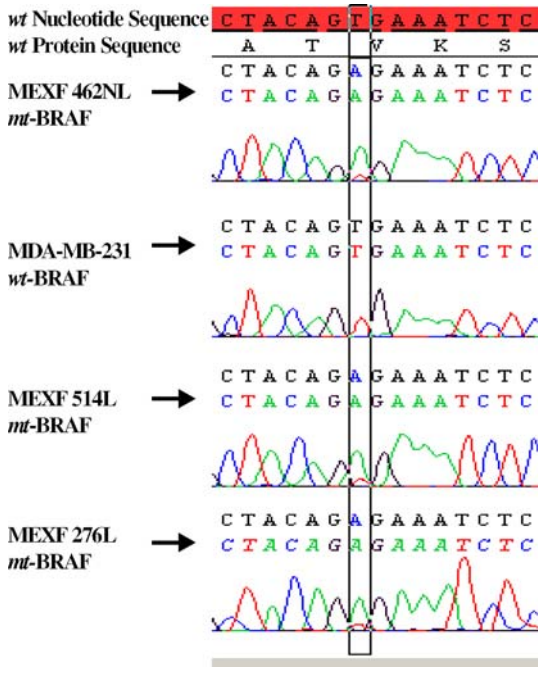

Fig. 4 Electropherograms of BRAF exon 15 sequences analyzed by SeqScape 2.0 software. The T1796A mutation leading to a V599E amino acid change is shown for the melanoma cell lines MEXF 462NL, MEXF 276L and MEXF 514L. The wild-type sequence represents that of the breast cancer cell line MDA-MB-231. The 1796 location of the T to A substitution in the genomic DNA sequence is indicated by the *box*. Nucleotide and protein sequences at the top represent wild-type BRAF from GenBank

comitantly cyclin D1. A rapid fall in AKT phosphorylation preceded any decline in AKT protein expression, particularly in drug-sensitive MEXF 276L cells and somewhat less in MEXF 462NL cells which showed an intermediate responsiveness (Fig. 3). In contrast, the resistant MEXF 514L cell line showed only a slight decrease in pAKT expression at a very late time point (96 h) in the case of both Hsp90 inhibitors; AKT was effected to the same extent as pAKT. The very early (2–4 h) and dramatic drop in pAKT levels after 17AAG/17DMAG treatment of sensitive MEXF 276L cells can be explained by the previous findings of Basso et al. [23] who demonstrated that AKT activation is dependent on erbB2/erbB3 heterodimer formation, which is only possible in this cell line. Thus, if erbB2/erbB3 heterodimers are disrupted by the actions of 17AAG, PI3K/AKT activation is blocked, thereby inhibiting crucial cell survival signals and hence cell growth [22].

The kinase partner of cyclin D1, cdk4, has also been implicated in the mode of action of Hsp90 modulators. Interestingly, its expression was reduced at earlier time points (4 h after initiation of treatment) in the cell lines with less sensitivity as compared to cells that were more sensitive (16 h after initiation of treatment). In all cell lines investigated, cyclin D1 protein expression decreased after cdk4 protein expression had declined, suggesting that cyclin D1 protein decreased as a result of a lack of cdk4 protein.

Craf-1, a well-studied client protein of Hsp90, decreased in all three melanoma cell lines after blockage of its chaperone Hsp90 with 17AAG/17DMAG, but at later time points and to the same extent (48–96 h). Its pattern of expression seems therefore not to be a determinant of 17AAG/17DMAG melanoma sensitivity. Nonetheless, the RAS/RAF/MAPK pathway is a critical component of tumor cell proliferation and survival in general, but specifically in melanoma [33]. BRAF, a member of the RAF family of serine-threonine kinases, activates the MAPK cascade, when bound to activated RAS. Recent studies have shown frequent BRAF mutations in melanoma cell lines and tissues [18, 27, 33]. The most frequent mutation in the BRAF gene in melanomas has been found in exon 15 where a T1796A substitution results in to a V599E amino acid change, and as a consequence kinase activity and tumorigenicity of BRAF mutant cells are increased [27]. In this context, it has been shown that 17AAG causes selective targeting of mutated BRAF to an NP-40-insoluble fraction of the proteasome, where it is degraded [34]. It is therefore tempting to hypothesize that the mutant BRAF status might have determined 17AAG/17DMAG sensitivity in our melanoma cell lines. That is, dependence on mutant BRAF activity might have rendered MEXF 276L cells more sensitive to Hsp90 depletion resulting in growth inhibition and apoptosis. The analyses of exon 15 genomic DNA sequences for BRAF revealed, however, that all melanoma cell lines used in this study harbored the V599E amino acid change. Hence, the responses of melanoma cell lines to 17AAG/17DMAG seen in this study cannot be attributed to mutant or wild-type BRAF status.

Although apoptosis was investigated by only one method, namely PARP cleavage, induction of cell death by this pathway occurred in all cell lines studied here, with 17AAG and 17DMAG at their respective TGI concentrations. These findings raise the hypothesis that while cell cycle arrest can lead to apoptosis irrespective of change in Hsp90 levels and the presence of erbB2, cell types expressing and/or overexpressing erbB2 with loss of erbB2 related to Hsp90 depletion, may be the most sensitive to the action of geldanamycin analog. Future examination of this possibility should be considered in preclinical and clinical studies.

In summary, 17DMAG is more potent than 17AAG. Both drugs appear to act by modulating Hsp90. This in turn leads to attenuation of several proteins that have been described as client proteins for Hsp90 and results in inhibition of various signal transduction pathways, e.g. the PI3K pathway. Therefore, further preclinical evaluation of 17DMAG as an Hsp90 modulator that may be superior to 17AAG is warranted. Combination studies with other agents as well as investigations into the mode(s) of Hsp90 depletion are underway. Overall, the results presented here make a strong case for an assessment of the water-soluble geldanamycin analog 17DMAG in clinical trials.

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