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Molecular determinants of cancer cell sensitivity and resistance towards the sesquiterpene farnesol

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Dedicated to Professor Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Farnesol is a non-cyclic sesquiterpene (isoprenoid) found in the essential oils of many plants. In cancer biology, farnesylation of mutated Ras oncoproteins allows the proteins to dock to the membrane and be functionalized. Therefore, farnesyltransferase is a target for drug development to inhibit Ras. Farnesol exhibits cytotoxic activity against tumor cells *in vitro* and *in vivo,* implying that novel treatment strategies may be devised independent of Ras farnesylation. Tumors frequently develop resistance towards standard chemotherapies, and thus novel agents are urgently required that bypass the cross-resistance evoked by established anticancer drugs. We investigated whether classical mechanisms of drug resistance such as ATP-binding cassette transporters (P-glycoprotein/MDR1, MRP1, BCRP), the tumor suppressor gene TP53, and the oncogene EGFR play a role in the response of tumor cells to farnesol. Remarkably, none of these genes conferred resistance to farnesol, indicating that this compound may be useful for the treatment of otherwise drug-resistant and refractory tumors expressing these mechanisms of resistance. Furthermore, we applied a pharmacogenomic approach to explore molecular determinants of sensitivity and resistance to farnesol. Among the candidates were genes involved in apoptosis (*STAB2, NUMBL*), regulation of transcription (*CDYL, FOXA2*) and diverse other functional groups (*INE1, CTRL, MRS2, NEB, LMO7, C9orf3, EHBP1*). The fact that these genes are not associated with resistance to traditional anticancer drugs suggests farnesol may possess a novel mechanism of action, and consequently might bypass drug resistance to established chemotherapeutics.

1. Introduction

Farnesol is a non-cyclic sesquiterpene (isoprenoid) found in the essential oils of a wide array of plants including lemongrass, rose, and many fruits and vegetables. It was first identified in the acacia tree [*Acacia farnesiana* L. (Willd.) also known as *Vachellia farnesiana* L. (Willd.)]. In plants, farnesol serves as a pheromone for insects and exerts antibacterial and antifungal activities (Levinson 1972). Isoprene, or 2-methyl-1,3-butadiene, serves as building block for terpenes. Farnesol is formed *via* the 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase pathway. Two activated isoprenes, dimethyl allyl pyrophospahe (DMAPP) and isopentenyl pyrophosphate (IPP), form geranyl pyrophosphate (GPP) in a reaction catalyzed by geranyl pyrophosphate synthetase. Subsequently, IPP (a 5 carbon molecule) is further processed to GPP (a 10 carbon molecule), to the sesquiterpene farnesyl pyrophosphate or FPP (a 15 carbon molecule), and to the diterpene geranylgeranyl pyrophosphate (a 20 carbon molecule) (Goldstein and Brown 1990). FPP is an intermediate in the biosynthesis of sesquiterpenes such as farnesene, which, when oxidized, becomes farnesol. Interestingly, two FPP molecules can react to form the cholesterol precursor squalene. Also, HMGCoA reductase inhibitors such as statins inhibit both isoprenoid and cholesterol biosynthesis (Edwards and Ericsson 1999).

In humans, farnesol activates the farnesyl X receptor (FXR) – a transcription factor regulating cholesterol and bile acid metabolism (Fayard et al. 2001). This may be one of the reasons why farnesol-containing fruits and vegetables exert favorable effects on cholesterol and bile acid homeostasis (Modica et al. 2010).

Isoprenoids play an important role in prenylation reactions. The attachment of prenyl groups to proteins mediates their anchorage in cellular membranes. In cancer biology, farnesylation and geranylation of mutated Ras oncoproteins allow membrane docking and, hence, functionalization (Jackson et al. 1990). Therefore, farnesyltransferase has been an important target to develop inhibitors against. The hope is that such inhibitors will reduce Ras function. Although potent farnesyltransferase inhibitors have been preclinically identified, their therapeutic efficacy was unclear in clinical studies for two main reasons. Firtly, tumors with mutated Ras oncoproteins may use geranyltransferase instead of farnesyltransferase as bypass mechanism for prenylation to functionalize Ras. Secondly, farnesyltransferase inhibitors kill tumors even in the absence of Ras mutations indicating Ras-independent mechanisms of action (Zhu et al.

Table 1: IC_{50} values of farnesol in a panel of cancer cell lines

Cell line	IC_{50} farnesol (µM)	Degree of resistance
CCRF-CEM	82.81±5.76	
CEM/ADR5000	165.38±2.20	2.0
HL60	94.75 ± 1.39	
HL60AR	194.00 ± 12.21	2.1
MDA-MB-231 pcDNA	120.05 ± 19.69	
MDA-MB-231BCRP	116.81 ± 11.28	0.97
HCT116 WT	98.17 ± 19.96	
HCT116 p53-/-	107.39 ± 18.07	1.1
U87MG	86.01 ± 3.64	
U87MG.ΔEGFR	103.54 ± 30.50	1.2

The degrees of resistance were calculated by dividing the IC_{50} value of each resistant cell line by the IC_{50} value of the corresponding parental cell line.

Table 2: Correlation of IC_{50} values for farnesol with gain of the chromosomal locus of the *MDR1* gene (7q21), expression of *MDR*1 mRNA, and accumulation of the P-glycoprotein/MDR1 substrate, rhodamine 123 (R123)

		Farnesol	Doxorubicin
Chromosomal gain of 7q21	R-value	<0.30	0.513
	P-value	0.088	3.05×10^{-5}
MDR1 mRNA (RT-PCR)	R-value	<0.30	0.379
	P-value	>0.05	0.003
MDR1 mRNA (microarray)	R-value	<0.30	0.551
	P-value	>0.05	3.02×10^{-6}
MDR1 mRNA (Northern blot)	R-value	<0.30	0.361
	P-value	>0.05	0.002
R123 accumulation	R-value	<0.30	0.454
	P-value	>0.05	1.50×10^{-4}

Doxorubicin was used as a positive control. Cut-off values were $R > 0.3$ and $P < 0.05$.

2003; Doll et al. 2004; Appels et al. 2005; Tsimberidou et al. 2010).

Farnesol exhibits considerable chemopreventive and cytotoxic activities against tumor cells *in vitro* and *in vivo* (Chaudhary et al. 2009). Farnesol may act as a starting point for the development of cytotoxic therapies independent of Ras farnesylation. Farnesol has been found to induce formation of reactive oxygen species, cause cell cycle arrest in the G0/G1 phase, induce apoptosis, activate MAPK pathways, and regulate transcription factors such as farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs), nuclear factor-kappaB (NF-kappaB) and others (Joo and Jetten 2010). These effects clearly indicate that farnesol's activity towards cancer cells is multi-faceted in nature. However, farnesol's specific molecular mechanism of action has not yet been fully elucidated. Thus far it is unknown whether tumor cells develop resistance to farnesol, as they do for most established anticancer drugs.

In the present investigation, we analyzed whether classical mechanisms of resistance towards established anticancer drugs, play a role in the cellular response to farnesol. The classical resistance mechanisms investigated were the ATP-binding cassette (ABC) transporters P-glycoprotein/*MDR1*, MRP1, and BCRP; the tumor suppressor TP53; and the epidermal growth factor receptor (EGFR). To gain a more comprehensive understanding of the molecular determinants of cancer cells' response to farnesol, we then performed bioinformatical COMPARE analysis and hierarchical cluster analyses of microarray-based transcriptomic mRNA expression data in a panel of 60 cell lines from the Developmental Therapeutics Program of the National Cancer Institute, USA (http://dtp.nci.nih.gov).

2. Investigations and results

2.1. Role of ABC-transporters in resistance to farnesol

In a resazurin assay, we analyzed multidrug-resistant P-glycoprotein/*MDR1*-overexpressing CEM/ADR5000 cells and their drug-sensitive parental cell line, CCRF-CEM, for their response towards farnesol. The IC_{50} values for farnesol were 165.40 µM in CEM/ADR5000 cells and 82.83 µM in CCRF-CEM cells (Table 1). The degree of resistance calculated from the IC_{50} values was 2.0, indicating a very low degree of cross-resistance of CEM/ADR5000 cells to farnesol. For comparison, CEM/ADR5000 cells are highly resistant to standard drugs such as doxorubicin (1036-fold), vincristine (613-fold), paclitaxel (438-fold) and others (Efferth et al. 2008).

To prove a possible role of P-glycoprotein/*MDR1* for resistance to farnesol in more detail, we investigated a panel of 60 tumor cell lines. We attempted to correlate DNA gain/amplification at the chromosomal locus of the *MDR1* gene (7q21) and mRNA expression of *MDR1* (as assayed by Northern blot, RT-PCR or microarray hybridization) with the IC_{50} values for farnesol in these cell lines. Furthermore, we looked for a relationship between accumulation of the fluorescent dye rhodamine 123 (R123) with the IC_{50} values for farnesol. R123 is a specific substrate of P-glycoprotein, and R123 accumulation can serve as an assay for P-glycoprotein function (Efferth et al. 1989; Sonneveld and Wiemer 1997). Using Pearson's rank correlation test, the IC_{50} values for farnesol did not correlate with any of these parameters (Table 2), indicating that farnesol is not involved in the P-glycoprotein/*MDR1*-mediated multidrug resistance phenotype. By contrast, the control drug doxorubicin, which is a well-known substrate of P-glycoprotein, significantly correlated with all of these parameters ($P < 0.05$; Table 2).

To see whether other ABC-transporters play a role for farnesol, we tested MRP1-overexpressing HL60/AR cells and BCRP-transfected MDA-MB-231 cells. HL60/AR cells were only 2.1-fold more resistant to farnesol than the drug-sensitive HL60 parent line and the MDA-MB-231/BCRP transfectants did not demonstrate increased resistance to farnesol compared to the non-transfected MDA-MB-231 cell line (Table 1).

2.2. Role of TP53 in resistance to farnesol

Since the tumor suppressor TP53 is another important factor in drug resistance, we analyzed the possibility that TP53 may affect tumor cell response to farnesol in TP53 knockout (p53–/–) and wild-type (p53+/+) HCT116 cells. As shown in Table 1, the IC_{50} values were 107.39 µM for HCT116 p53–/– and 98.17 µM for HCT116 p53+/+ cells, resulting in a degree of resistance of 1.09. Hence, HCT116 p53–/– were not significantly more resistant to farnesol than HCT116 p53+/+ cells. To corroborate this result in a larger panel of cell lines, we correlated the mutational status of 60 tumor cell lines with their IC_{50} values for farnesol. Again, no significant relationships were found (Fig. 1), indicating that p53 does not influence resistance or sensitivity of tumor cells to farnesol.

2.3. Role of EGFR in resistance to farnesol

EGFR is known to mediate drug resistance (Volm et al. 1992; Konkimalla et al. 2007). Therefore, we compared farnesol's activity in U87MG cells transfected with a mutation-activated *EGFR* cDNA (U87MG.Δ EGFR) to its activity in

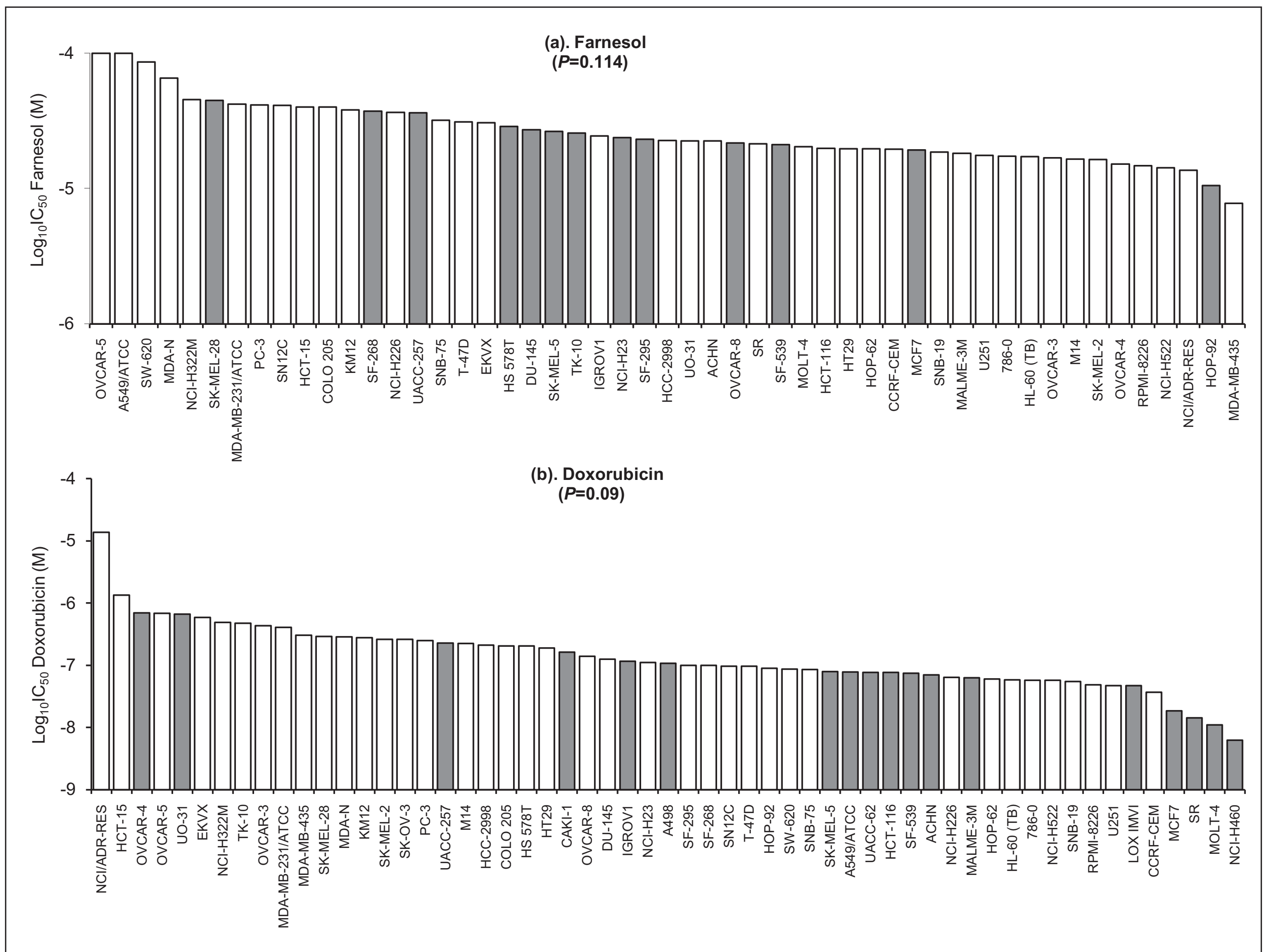

Fig. 1: Ranked order of log10(IC_{50}) values for (a) farnesol and (b) doxorubicin (as a positive control) in cell lines included in the screening panel of the Developmental Therapeutics Program (NCI, USA) in comparison to the mutational status of the tumor suppressor gene TP53 (solid bars: wild-type, open bars: mutated TP53).

non-transfected U87MG control cells. The IC_{50} values were 86.03 μM and 103.57 μM, respectively, resulting in a degree of resistance of 1.2 (Table 1). Thus, EGFR does not affect cellular responsiveness to farnesol. This result was confirmed using the panel of 60 tumor cell lines. There was no significant correlation between the expression of EGFR mRNA or protein and the IC_{50} values for farnesol (Table 3).

2.4. Microarray, COMPARE, and cluster analyses

We took a pharmacogenomic approach to further explore the molecular determinants of sensitivity and resistance to farnesol. Accordingly, we mined the transcriptome-wide mRNA expression database of the NCI and correlated the data with the IC_{50} values for farnesol to identify putative molecular determinants of cellular response to farnesol.

Standard COMPARE analyses were performed to identify genes whose expression was correlated with resistance to farnesol. Conversely, we also conducted reverse COMPARE analysis to find factors associated with sensitivity. Only variables with a correlation coefficient of R>0.6 (standard COMPARE) or R< –0.6 (reverse COMPARE) were considered (Table 4). Among the candidates were genes involved in apoptosis (*STAB2, NUMBL*), transcriptional regulation (*CDYL, FOXA2*) and diverse other functional groups (*INE1, CTRL, MRS2, NEB, LMO7*, *C9orf3*, *EHBP1*). A number of other transcripts identified by the COMPARE analyses have not yet been characterized in terms of function.

Table 3: Correlation of IC_{50} values for farnesol with EGFR mRNA and protein expression

		Farnesol	Erlotinib
EGFR mRNA (RT-PCR slot blot)	R-value	<0.30	–0.244
	P-value	>0.05	0.031
EGFR mRNA (microarray, Affimetrix U95Av2)	R-value	<0.30	–0.390
	P-value	>0.05	0.001
EGFR mRNA (microarray, Affimetrix U133A and B)	R-value	<0.30	0.419
	P-value	>0.05	5.27×10^{-4}
EGFR protein (protein lysate array)	R-value	<0.30	–0.376
	P-value	>0.05	0.002

Erlotinib was used as a positive control drug. Cut-off values were $R > 0.3$ and $P < 0.05$.

Table 4: Correlation of constitutive mRNA expression of genes identified by COMPARE analyses with IC_{50} values of farnesol for 60 tumor cell lines

COMPARE coeficient	Experimental ID	GenBank Accession	Symbol	Name	Function
Standard COMPARE:					
0.584	GC51668	AA884400	none	none	unknown
0.534	GC94411	R92610	STAB2	stabilin 2	engulfment of apoptotic cells
0.533	GC78930	AI932342	none	none	unknown
0.522	GC60676	AI207040	CDYL	chromodomain protein, Y-like	histone transferylacetase, transcriptional repressor
0.516	GC101309	Y10696	INE1	inactivation escape 1 (non-protein coding)	EST transcript from a gene escaping inactivation
0.513	GC175967	BF508685	CTRL	chymotrypsin-like	serine-type endopeptidase
0.513	GC55956	AF052167	MRS2	MRS2 magnesium homeostasis factor homolog (S. cerevisiae)	magnesium transporter
0.508	GC59876	AI150628	NEB	nebulin	oxidoreductase, structural constituent of muscle
0.501	GC101709	Z78347	NUMBL	NUMB homologue (*Drosophila*)-like	negative regulator of NF-kappa-B signaling pathway, neurogenesis
0.493	GC50778		none	none	unknown
Reverse COMPARE:					
–0.649	GC54800	AB028021	FOXA2	Forkhead box A2	transcription factor
–0.62	GC28633	AB024057	none	none	unknown
–0.566	GC44605	AA451859	none	none	unknown
–0.566	GC63375	AI344189	none	none	unknown
–0.563	GC31821	AJ001403	none	none	unknown
–0.563	GC31803	Y09788	none	none	unknown
–0.561	GC38356	AB020665	LMO7	LIM domain 7	ubiquitin-protein ligase
–0.557	GC33217	M74587	none	none	unknown
–0.555	GC31645	AF043897	C9orf3	chromosome 9 open reading frame 3	aminopeptidase
–0.546	GC30853	AB020710	EHBP1	EH domain binding protein 1	actin reorganization

Only genes with correlation coefficients of >0.6 or <–0.6 were considered. Positive correlation coefficients indicate direct correlations to $\log_{10}IC_{50}$ values, negative ones indicate inverse correlations. Information on gene functions was taken from the OMIM database, NCI, USA (http://www.ncbi.nlm.nih.gov/Omim/) and from the GeneCard database of the Weizman Institute of Science, Rehovot, Israel (http://bioinfo.weizmann.ac.il/cards/index.html).

Next, the genes identified by standard and reverse COMPARE analyses were subjected to hierarchical cluster analysis. The dendrogram identified by this procedure can be divided in three major branches (Fig. 2). The distribution of sensitive and resistant cell lines differed significantly between the branches of the dendrogram ($P = 0.003$, Chi^2-test) (Table 5).

3. Discussion

Farnesol, a sesquiterpene, exerts cytotoxicity towards a panel of cell lines of different tumor types. Sesquiterpenes are known for their pharmacological activities, including anti-inflammatory, anti-microbial, and anticancer activities (McGovern et al. 1999; Copp 2003; Merfort 2011; Chen et al. 2011). As tumors frequently develop resistance towards standard chemotherapies, novel agents that bypass cross-resistance evoked by established anticancer drugs are urgently required. In the present investigation, we examined whether classical mechanisms of drug resistance, *e.g.* ABC transporters, the tumor suppressor TP53, and the oncogene EGFR, play a role in tumor cell response to farnesol. Interestingly, we found that none of these three mechanisms of drug resistance confer resistance to farnesol. This indicates that farnesol may be useful in the treatment of otherwise drug-resistant and refractory tumors expressing these mechanisms of resistance. To the best of our knowledge, the present investigation is the first to report on the topic of ABC transporters, mutated TP53, and EGFR in the context of cancer treatment with farnesol. In microorganisms, farnesol is known to inhibit ABC transporter–mediated drug efflux (Jin et al. 2010; Sharma and Prasad 2011). Whether farnesol might exert a similar function in human ABC transporters is unknown as of yet. Despite extensive efforts to find inhibitors of P-glycoprotein and other ABC transporters to overcome multidrug resistance in tumors, clinical results with P-glycoprotein inhibitors have been rather disappointing (Fischer et al. 1998; Kruijitzer et al. 2002; Fracasso et al. 2004). Therefore, the focus has shifted to searching for compounds that are not substrates for ABC transporters. Such substances can theoretically kill multidrug-resistant tumor cells with efficacies similar to drug-sensitive cells. In our investigation, farnesol revealed only a two-fold degree of resistance in multidrug-resistant CEM/ADR5000 cells with high degrees of resistance to doxorubicin (1036-fold), vincristine (613-fold), docetaxel (438-fold) and other established anticancer drugs (Efferth et al. 2008). In a panel of 60 cell lines of different tumor origin, no correlation was found between the IC_{50} values for farnesol and expression and activity of P-glycoprotein. This indicates that farnesol is either a weak substrate or is not

Table 5: Separation of clusters of 60 NCI cell lines obtained by hierarchical cluster analysis shown in Fig. 2 in comparison to drug sensitivity

	Partition	Cluster 1	Cluster 2	Cluster 3
sensitive	< -4.649 M	0	6	19
resistant	> -4.649 M	3	18	6
chi-Squared Test	$P = 0.003$			

The median $\log_{10}IC_{50}$ value (–4.649 M) for each compound was used as cut-off to separate tumor cell lines as being "sensitive" or "resistant".

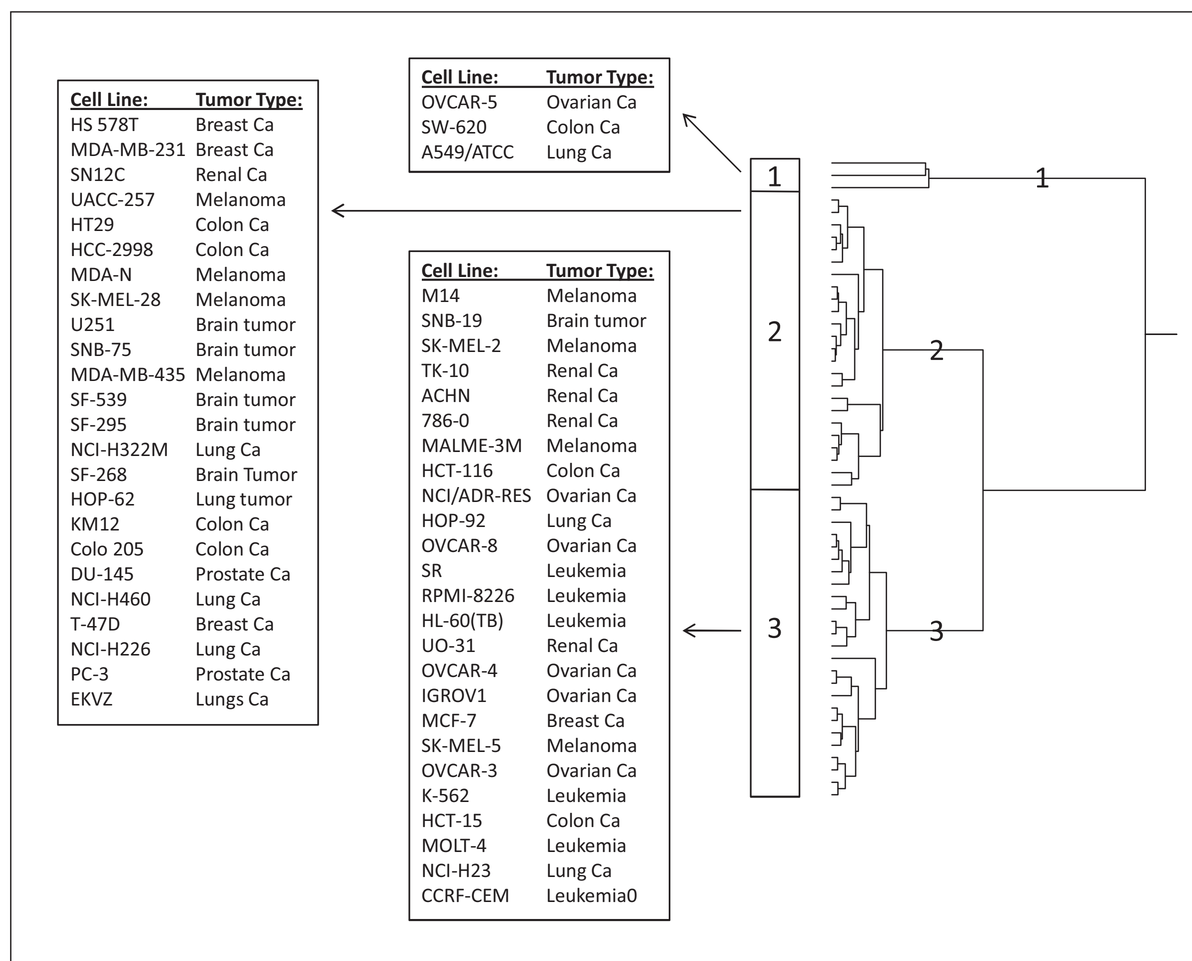

Fig. 2: Dendrogram of hierarchical cluster analysis (WARD method) obtained from microarray-based mRNA expression profiles of genes correlating with farnesol. The dendrogram shows the clustering of 60 NCI cell lines.

a substrate of this drug pump. Likewise, no significant relationships were found between IC_{50} values for farnesol and the expression of MRP1 and BCRP, two other ABC transporters. This data suggests that farnesol has great potential for killing multidrug-resistant tumors overexpressing ABC-transporters.

Drug resistance is multi-faceted and is determined by several factors. Loss-of-function mutations in TP53 can also cause resistance towards established anticancer drugs (El-Deiry 2003; Cimoli et al. 2004; Lai et al. 2012). Therefore, we analyzed whether TP53 knock-out cells were more resistant to farnesol than were TP3 wild-type cells. In HCT116p53+/+ and HCT116–/– cells, we observed that farnesol was cytotoxic toward both cells lines with similar IC_{50} values. In a panel of 60 tumor cell lines, no correlation was found between the mutational status of TP53 and the IC_{50} values for farnesol. Hence, farnesol is active in TP53-mutated tumor cells.

Oncogenes not only contribute to carcinogenesis, but also to resistance towards chemo- and radiotherapy (El Deiry 1997). Therefore, we investigated whether EGFR, one of the most important oncogenes in human cancers, plays a role in the response of tumor cells to farnesol. It has been shown that mutations in the *EGFR* gene not only cause resistance towards EGFR-directed small molecules and antibodies (Bonanno et al. 2011; Brand et al. 2011; Kosaka et al. 2011), but also towards established cytostatic drugs (Volm et al. 1992; 1993; Navolanic et al. 2003). In our own experiments, we found that U87MG cells transfected with mutation-activated *EGFR* cDNA generally possess increased resistance to cytotoxic natural products as compared to non-transfected U87MG cells. (Efferth et al. 2003a,b). However, EGFR does not affect cellular sensitivity to farnesol.

Furthermore, we analyzed molecular determinants of tumor cell sensitivity and resistance towards farnesol. By microarray-based gene expression and COMPARE analyses, we correlated the IC_{50} values for the compound with transcriptomic mRNA expression levels in a panel of 60 tumor cell lines (Scherf et al. 2000). This approach has been successfully used to elucidate the mode of action of novel compounds in the past (Leteurtre et al. 1993). Cluster and COMPARE analyses are also useful for comparing gene expression profiles with IC_{50} values for investigational drugs to identify candidate genes involved in drug resistance (Efferth et al. 2003c) and for patient prognoses in clinical oncology (Volm et al. 2002). We identified genes closely associated with the response to farnesol that belonged to diverse functional groups such as transcription factors (*CYDL, FOXA2, NUMBL*), genes involved in metabolism (*CTRL*, *C9orf3*), apoptosis (*STAB2*), and others. Although these genes have not, in the past, been associated with drug resistance, this approach identified several novel candidate genes that were significantly associated with sensitivity or resistance to farnesol. These results merit further investigation to prove the contribution of these genes to farnesol resistance.

The fact that the genes associated with sensitivity and resistance to farnesol are not associated with resistance to classical anticancer drugs indicates that farnesol may possess a novel mode of action and further strengthens the hypothesis that farnesol can bypass traditional drug resistance mechanisms. The genes identified by microarray and cluster analyses were from

diverse functional groups, indicating that farnesol may exert its cytotoxic effects via a multiplicity of mechanisms. The phrase "multiplicity of mechanisms" can mean that farnesol either has multiple targets leading to multiple effects, or has one target that leads to activation and/or inactivation of multiple molecules downstream of this target. Multi-specificity is a general feature of natural products. Rather than acting on one single target, multiple targets and pathways are affected. Multi-specificity prevents the development of resistance towards a single bioactive compound, which likely was an important selection advantage during evolution (Efferth and Koch 2011).

4. Experimental

4.1. Cell lines

4.1.1. Multidrug-resistant tumor cell lines

Leukemic CCRF-CEM and HL60 cells were maintained in RPMI 1640 medium (Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passaged twice weekly. All experiments were performed on cells in the logarithmic growth phase. The P-glycoprotein/*MDR1*-overexpressing CEM/ADR5000 cell line was maintained in 5000 ng/ml doxorubicin. The *MRP1*-expressing HL60/AR subline was continuously treated with 100 nM daunorubicin. The establishment of the resistant sublines has been previously described (Kimmig et al. 1990; Brügger et al. 1999). Sensitive and resistant cells were kindly provided by Dr. J. Beck (Department of Pediatrics, University of Greifswald, Greifswald, Germany). Breast cancer cells transduced with a control vector (MDA-MB-231-pcDNA3) or with cDNA for the breast cancer resistance protein *BCRP* (MDA-MB-231-BCRP clone 23) were maintained under standard conditions as described above for CCRF-CEM and HL60 cells. Generation of the cell lines followed a published protocol (Doyle et al. 1998). The cell lines were continuously maintained in 800 ng/ml geneticin (Invitrogen, Karlsruhe, Germany). The mRNA expression of *MDR1*, *MRP1*, and *BCRP* in the resistant cell lines has been reported (Efferth et al. 2003a; Gillet et al. 2006).

4.1.2. Knockout cell lines

Human wild-type HCT116 colon cancer cells (p53+/+) as well as knockout clones (p53–/–) derived by homologous recombination (Bunz et al. 1998) were a generous gift from Dr. B. Vogelstein and H. Hermeking (Howard Hughes Medical Institute, Baltimore, MD). The cell lines were propagated in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin (Invitrogen). The cells were passaged twice weekly.

4.1.3. Transfected cell line

The establishment of human glioblastoma multiforme U87MG cells transduced with an expression vector harboring an epidermal growth factor receptor (*EGFR*) gene with a deletion of exons 2 through 7 (U87MG.ΔEGFR) has been reported previously (Huang et al. 1997). Transduced and non-transduced cell lines were kindly provided by Dr. W. K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA) and cultured as described (Nagane et al. 1996).

4.1.4. Cell lines of the developmental therapeutics program of the NCI

The panel of 60 human tumor cell lines of the Developmental Therapeutics Program of the National Cancer Institute (NCI, USA) consisted of leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian cancer, breast cancer, and prostate carcinoma cells as well as tumor cells of the central nervous system. Their origin and processing have previously been described (Alley et al. 1988). Cells were assayed by means of a sulforhodamine B assay (Rubinstein et al. 1990).

4.1.5. Resazurin cell growth inhibition assay

Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay (O'Brien et al. 2000) was used to assess the cytotoxicity of the studied samples. The assay tests cellular viability and mitochondrial function. Briefly, adherent cells were grown in tissue culture flasks, and then harvested by treating the flasks with 0.025% trypsin and 0.25 mM EDTA for 5 min. Once detached, cells were washed, counted and an aliquot (5×10^3 cells) was placed in each well of a 96-well cell culture plate in a total volume of 100 μl. Cells were allowed to attach overnight and were then treated with the samples. After 48 h, 20 μl of 0.01% w/v resazurin solution was added to each well and the plates were incubated at 37 °C for 1–2 h. Fluorescence was measured on an automated 96-well Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. For leukemia cells, aliquots of 5×10^4 cells/ml (obtained from overnight suspension) were seeded in 96-well plates, and extracts were added immediately. After 72 h incubation, plates were treated with resazurin solution as described above. Doxorubicin was used as positive control. Each assay was conducted at least three times, with two replicates each. Cell viability was evaluated based on a comparison with untreated cells. IC_{50} values were taken to be the concentration of sample required to inhibit 50% of cell proliferation, and were calculated from a calibration curve by linear regression using Microsoft Excel.

4.1.6. Statistical analysis

Data from mRNA microarrays of the NCI tumor cell line panel is available (Scherf et al. 2000; Staunton et al. 2001) through the NCI website (http://dtp.nci.nih.gov). For hierarchical cluster analysis, objects were classified by calculating distance according to the closeness of between-individual distances by means of the Ward method. All objects were assembled into cluster trees (dendrograms). Cluster models have previously been validated for gene expression profiling and for approaching molecular pharmacology of cancer (Efferth et al. 1997; Scherf et al. 2000). Hierarchical cluster analyses applying the complete linkage method were performed with the WinSTAT program (Kalmia, Cambridge, MA, USA). Missing values were automatically omitted by the program, and the closeness of two joined objects was calculated by the number of data points they contained. In order to calculate distances between all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean = 0 and a variance = 1.

COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the NCI cell lines. The methodology has previously been described in detail (Paull et al. 1989). Briefly, every gene of the NCI microarray database was ranked for similarity of its mRNA expression to the $\log_{10}IC_{50}$ values for farnesol To derive COMPARE rankings, a scale index of correlation coefficients (R-values) was created. In the standard COMPARE approach, greater mRNA expression in cell lines correlates with enhanced drug resistance, whereas in reverse COMPARE analysis, increased mRNA expression indicates drug sensitivity.

Pearson's correlation test was used to calculate significance values and rank correlation coefficients as a relative measure of the linear dependency of two variables. This test was implemented using the WinSTAT Program (Kalmia). The Pearson correlation test was used as a measure for interval-scaled linear correlations. We used Pearson's instead of Spearman's rank correlation test because Spearman's test is based on the equidistance of values, and the values used for our analyses were not equidistant.

The chi-squared test was applied to bivariate frequency distributions of pairs of nominal scaled variables. It was used to calculate significance values (*P*-values) and rank correlation coefficients (R-values) as a relative measure of the linear dependency of two variables. This test was implemented using the WinSTAT program (Kalmia Co.). The chi-squared test determines the difference between each observed and theoretical frequency for each possible outcome, squares them, divides each by the theoretical frequency, and than takes the sum of the results. Performing the chi-squared test necessitated defining cell lines as being sensitive or resistant to the test compounds. This was done by taking the median IC_{50} value as a cut-off threshold.

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