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Identification of gene expression profiles predicting tumor cell response to L-alanosine

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Abstract

The methylthioadenosine phosphorylase (*MTAP*) gene gained considerable interest as therapeutic target for tumors with the 9p21 deletion. This gene maps to 9p21 and loss of this chromosomal region in tumors offers an unique opportunity for chemoselective treatment, since MTAP is an important salvage enzyme for the formation of adenine that is needed for DNA synthesis. L-Alanosine, an antibiotic from *Streptomyces alanosinicus*, blocks the common *de novo* purine biosynthesis pathway and, thereby, inhibits tumor cells with MTAP deficiency. Normal cells escape the detrimental effects of L-alanosine due to their proficiency in the MTAP salvage pathway. The present analysis was undertaken to gain insights into the molecular architecture of tumor cells that determines the response to L-alanosine apart from the *MTAP* gene. Analysis of cell doubling times and IC_{50} values for L-alanosine showed that slowly growing cell lines were more resistant to L-alanosine than rapidly growing ones. Mining the database of the National Cancer Institute (N.C.I.), for the mRNA expression of 9706 genes in 60 cell lines by means of Kendall's τ-test, false discovery rate calculation, and hierarchical cluster analysis pointed to 11 genes or expressed sequence tags whose mRNA expression correlated with the IC_{50} values for L-alanosine. Furthermore, we tested L-alanosine for cross-resistance in multidrug-resistant cell lines which overexpress selectively either the P-glycoprotein/*MDR1* (CEM/ADR5000), *MRP1* (HL-60/AR), or *BCRP* (MDA-MB-231-BCRP) genes. None of the multidrug-resistant cell lines was cross-resistant to L-alanosine indicating that L-alanosine may be suitable to treat multidrug-resistant, refractory tumors in the clinic. Finally, the IC_{50} values for L-alanosine of the 60 cell lines were correlated to the p53 mutational status and expression of p53 downstream genes. We found that p53 mutated cell lines were more resistant to L-alanosine than p53 wild type cell lines.

Keywords: Hierarchical cluster analysis; L-Alanosine; mRNA expression profiling; Multidrug resistance; p53 tumor suppressor

1. Introduction

In spite of all the progress made in cancer chemotherapy during the past decades, the cure from the disease is still not a reality for the majority of patients. Two major reasons for this are the development of drug resistance in tumor cells and the severe adverse side effects of most anticancer drugs. Enormous efforts have been undertaken to advance new treatment strategies to improve survival rates of cancer patients. The problem of distinction between normal and cancerous tissues is, however, still unsatisfactorily resolved.

A plethora of molecular alterations in tumor cells have been described with the aim of opening new avenues in cancer diagnostics and therapy. Among the most important genetic aberrations noted are alterations at the chromosomal locus 9p21. Deletions, loss of heterozygosity, mutations, and aberrant DNA methylation at this locus have been found in some 30 tumor entities, to date [1,2]. Several genes map to this locus: the tumor suppressor genes *CDKN2A* ($p16^{INK4a}$), the alternatively spliced $p14^{ARF}$, and *CDKN2B* ($p15^{INK4b}$), a cluster of interferon genes,

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Abbreviations: BCRP, breast cancer resistance protein; ESTs, expressed sequence tags; FDR, false discovery rate; N.C.I., National Cancer Institute; MDR, multidrug resistance; *MDR1*, multidrug resistance gene 1; MRP1, multidrug resistance-related protein 1; MTAP, methylthioadenosine phosphorylase.

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and the methylthioadenosine phosphorylase (*MTAP*) gene. The *MTAP* gene is frequently co-deleted with the *CDKN2A* ($p16^{INK4a}$) gene that is in close vicinity [3–8]. A survey of the literature shows that the *MTAP* gene is deleted in nearly 30% of those tumor types where 9p21 deletions occur [5–12]. The *MTAP* gene product has gained considerable interest as therapeutic target, as it is an important salvage enzyme for adenine and methionine. *MTAP* cleaves 5′-deoxy-5′-methylthioadenosine (MTA) into adenine and 5′-methylthioribose-1-phosphate (MTR-1-P). Adenine is then efficiently salvaged to form adenosine monophosphate (AMP). On the other hand, AMP is generated *via* the *de novo* purine biosynthesis pathway. AMP provides a source of adenine nucleotides for DNA synthesis. The deficiency of *MTAP* in tumor cells offers a unique opportunity to develop a selective therapy that spares normal cells. In *MTAP*-negative cells, the salvage of adenine by MTA is blocked, leaving *de novo* synthesis the only pathway for adenine nucleotide production. Blocking of the *de novo* synthesis pathway by antimetabolites such as methotrexate or L-alanosine kills efficiently *MTAP*-deficient tumor cells. Normal cells are rescued due to their capacity for adenine production *via* the MTAP salvage pathway. Methotrexate has been used for several decades in clinical oncology. Its curative value is, however, frequently hampered by the rapid emergence of drug resistance due to amplification of the dihydrofolate reductase gene and other mechanisms of resistance [13].

This focused our interest on L-alanosine, an antibiotic from *Streptomyces alanosinicus*. Its metabolite, L-alanosyl-5-amino-4-imidazole carboxylic acid ribonucleotide, is a strong inhibitor of adenosylsuccinate synthetase that converts inosinate (IMP) to AMP with high specificity. L-Alanosine has antineoplastic and antiviral activity (http://dtp.nci.nih.gov/mtargets_index.html; [14]). Recent microarray studies with other cytostatic drugs of natural origin, i.e. vincristine and vinblastine from *Vinca major* or paclitaxel from *Taxus brevifolia* showed that other thus far unrecognized mechanisms of action have to be considered in addition to the known target site of these drugs, e.g. microtubules [15–17]. Though the primary target of L-alanosine, adenosylsuccinate synthetase, has been identified [18], the response of tumor cells to anticancer drugs is likely to be multifactorial as well. The entire molecular architecture that determines the response of tumor cells to L-alanosine is unknown as of to date.

In the present investigation, we compared the inhibitory concentration 50% (IC_{50}) values in 60 cell lines of the screening panel of the Developmental Therapeutics Program of the N.C.I., as determined by the sulforhodamine assay [19] with the cell doubling times and with baseline mRNA expression levels of 9706 genes obtained by microarray analyses (http://www.dtp.nci.nih.gov/mtargets_index.html; [20,21]). We subjected these data to COMPARE analysis, Kendall's τ-test, FDR calculation, and hierarchical cluster analysis to further characterize mRNA expression profiles that correlate to cellular responses of these tumor cell lines to L-alanosine.

Many antitumor drugs of natural origin (e.g. taxanes, *Vinca* alkaloids, epipodophyllotoxins) including drugs from *Streptomyces* species (e.g. anthracyclines) are substrates of MDR transporters [22]. As L-alanosine is of natural origin as well (*S. alanosinicus*), we have addressed the question of whether L-alanosine is a substrate for the multidrug resistance transporters P-glycoprotein/*MDR1*, *MRP1*, and *BCRP*.

2. Material and methods

2.1. Drugs

L-Alanosine was kindly provided by the Drug Synthesis and Chemistry Branch, Chemotherapeutic Agents Repository, National Cancer Institute.

2.2. Cell lines

2.2.1. Multidrug-resistant tumor cell lines

Leukemic CCRF-CEM and HL-60 cells were maintained in RPMI medium (Gibco) supplemented with 10% FCS in a humidified 5% CO_2 atmosphere at 37 °C. Cells were passaged twice weekly. All experiments were performed with cells in logarithmic growth phase. The P-glycoprotein/*MDR1*-expressing CEM/ADR5000 subline was maintained in 5000 ng/mL doxorubicin. The MRP1-expressing HL-60/AR subline was continuously treated with 100 nM daunorubicin. The establishment of these resistant sublines has been previously described [23,24]. Sensitive and resistant cells were kindly provided by Dr. J. Beck (Department for Pediatrics, University of Tübingen). Breast cancer cells transduced with control vector (MDA-MB-231-pcDNA3) or with breast cancer resistance protein (*BCRP*) cDNA (MDA-MB231-BCRP clone 23) were maintained under standard conditions as described above for CCRF-CEM and HL-60 cells. The generation of the cell lines followed a published protocol [25]. The stably transduced cell lines were continuously maintained in 800 ng/mL G418. The cell lines have been tested to be mycoplasma-free using the DNA fluorochrome stains 4′-6-diamidino-2-phenyl-indole (DAPI; Sigma-Aldrich) or bisbenzimidazole (Hoechst 33258, Sigma-Aldrich) as described [26,27].

2.2.2. Cell lines of the N.C.I.

The origin and processing of the cell lines of the Developmental Therapeutics Program of the N.C.I. were previously described [28]. The panel for the present investigations consisted of 60 human tumor cell lines representing leukemia (CCRF-CEM, HL-60, K-562, MOLT-4, RPMI-8226, SR), melanoma (LOX-IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257, UACC-62), non-small cell lung cancer (A549/ATCC,

EKVX, HOP-62, HOP-92, N.C.I.-H226, N.C.I.-H23, N.C.I.-H322M, N.C.I.-H460, N.C.I.-H522), colon cancer (COLO205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620), renal cancer (786-0, A498, ACHN, CAKI-1, RXF 393, SN12C, TK-10, UO-31), ovarian carcinoma (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3), tumors of the central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251), prostate carcinoma (PC-3, DU-145), and breast cancer (MCF-7, N.C.I.-ADR Res, MDA-MB-231, HS578T, MDA-MB-435, MDA-N, BT-549, T-47D).

2.3. Growth inhibition assay

The *in vitro* response to cytostatic drugs was evaluated by means of a growth inhibition assay [29,30]. Aliquots of 5×10^5 cells/mL were seeded in culture medium, and drugs were added immediately at different concentrations. Cells were counted up to 10 days after seeding depending on the growth rate of the different cell lines. The resulting growth curves represent the net outcome of cell proliferation and cell death. Cell numbers were determined each in eight independent determinations.

2.4. Statistical analyses

Hierarchical cluster analysis is an explorative statistical method which groups at first sight heterogeneous objects into clusters of homogeneous objects. Objects are classified by calculation of distances according to the closeness of between-individual distances. All objects are assembled into a cluster tree (dendrogram). The merging of objects with similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relation. The procedure continues to aggregate clusters until there is only one. The distance of subordinate clusters to a superior cluster represents a criterion for the closeness of clusters as well as for the affiliation of single objects to clusters. Thus, objects with tightly related features appear together, while the distance in the cluster tree increases with progressive dissimilarity. The applicability of hierarchical cluster analysis for microarray data has been demonstrated previously [21]. Cluster analyses applying the complete-linkage method were done by means of the WinSTAT program (Kalmia). 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 of all variables included in the analysis, the program automatically standardizes the variables by transforming the data with mean = 0 and variance = 1.

COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the 60 cell lines of the N.C.I. Every gene of the Microarray Database of the N.C.I., is ranked for similarity of its mRNA expression to the IC_{50} values for L-alanosine. To derive COMPARE rankings, a scale index of correlations coefficients (*R*-values) is created. In the standard COMPARE approach, greater mRNA expression in cell lines correlate with enhanced drug sensitivity, whereas in reverse COMPARE analyses greater mRNA expression in cell lines indicate with drug resistance. The methodology has been described [31].

Kendall's τ-test was used to calculate significance values (*P*-values) and rank correlation coefficients (*R*-values) as a relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT program (Kalmia). Kendall's τ-test determines the correlation of rank positions of values. Ordinal or metric scaling of data is suited for the test and are transformed into rank positions. There is no condition regarding normal distribution of the data set for the performance of Kendall's τ-test.

Mann–Whitney's *U*-test is suited to analyze two rows of measured values for their significant differences. This test is a parameter-free procedure which does not need a normal distribution. Mann–Whitney's *U*-test was used as an implement of the WinSTAT program (Kalmia).

In addition to the calculation of *P*- and *R*-values, the problem of multiple hypothesis testing was addressed. The probability of type I errors increases, as the number of tests increases [32,33]. Therefore, a step-up resampling multicomparison procedure was applied to control the false discovery rate among the significant correlations at significance levels of 0.01 and 0.001, respectively. This program is available on http://www.math.tau.ac.il. The FDR is the expected proportion α of erroneous rejections among all rejections of the null-hypothesis [34].

3. Results

3.1. Effect of proliferative activity on cellular response to L-alanosine

We correlated the IC_{50} values for L-alanosine with the cell doubling times of the 60 N.C.I. cell lines as a parameter for cellular proliferation. Cell lines with longer cell doubling times were generally less responsive to L-alanosine than cell lines with shorter doubling times (Fig. 1). As analyzed by Kendall's τ-test, this relationship was statistically significant ($P = 0.0000246$; $R = 0.35961$). Thus, rapidly growing cell lines were more sensitive to L-alanosine than slowly growing ones.

3.2. mRNA expression profiling, FDR, and hierarchical cluster analysis

To gain insight into the mode of action of L-alanosine, we performed COMPARE analysis of the IC_{50} values for L-alanosine and the mRNA expression of 9706 genes in the 60 N.C.I. cell lines. The mRNA expression has been previously reported [21] and deposited in the N.C.I.'s

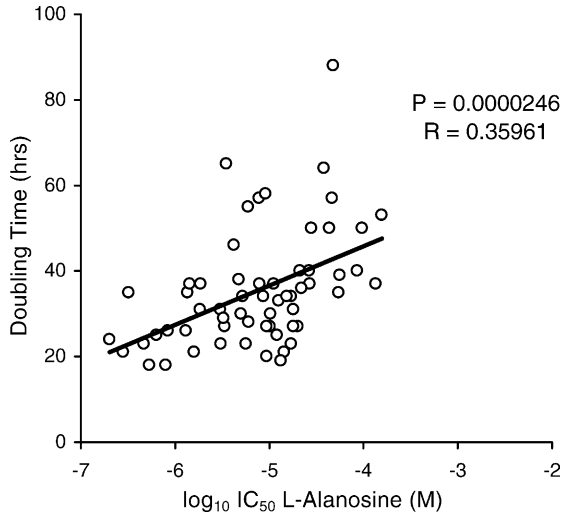

Fig. 1. Linear regression of cell doubling times of cell lines included in the screening panel of the Developmental Therapeutics Program (N.C.I.) in comparison to the $\log_{10}$ IC_{50} values for L-alanosine. Significance level (P) and correlation coefficient (R) were calculated using Kendall's τ-test.

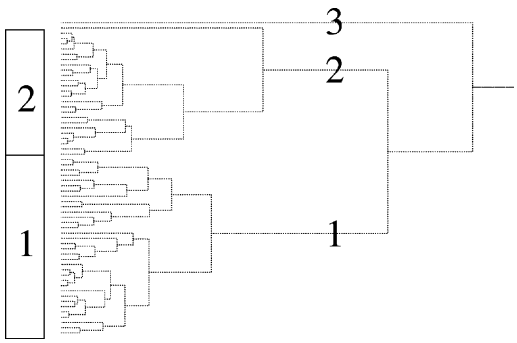

Fig. 2. Dendrogram obtained by hierarchical cluster analysis (complete linkage method) for mRNA expression of 11 genes or ESTs. The dendrogram shows the clustering of cell lines of the N.C.I.'s screening panel.

database (http://dtp.nci.nih.gov/mtargets/mt_index.html). First, a standard COMPARE analysis was performed. Cell lines that were most inhibited to L-alanosine (lowest IC_{50} values) were correlated with the highest mRNA expression levels of genes. Then, a reverse COMPARE analysis was done that correlated the most inhibited cell lines with the lowest gene expression level. Hence, genes correlating to drug sensitivity were identified by standard COMPARE, whereas genes correlating to L-alanosine resistance were found by reverse COMPARE. We used each 25 genes or ESTs with the highest correlation coefficients of both COMPARE analyses for subsequent FDR calculation. Adjusting the significance level to 0.01 revealed an α-value of 0.03201 which means that 49 of the 50 genes or ESTs with $P < 0.03201$ have a probability of 1% to correlate erroneously to cellular response to L-alanosine. If the significance level was adjusted to 0.001, 11 of 50 genes or ESTs have an error probability of 0.1% ($\alpha = 0.000184$). These 11 genes or ESTs were listed in Table 1. The *MTHFD2* gene was the only gene from the standard COMPARE analysis in this list, while the other 10 genes or ESTs were from the reverse COMPARE analysis.

These 11 genes or ESTs were then subjected to hierarchical cluster analysis using the complete linkage method. Following the dendrogram in Fig. 2 from the right side to the left one, two major branches of the cluster tree can be separated from each other with 34 and 25 cell lines, respectively. A third cluster branch contained only one cell line that was not further analyzed. The mean values (±SEM) of the mRNA expression of the 11 genes or ESTs in these two clusters are shown in Table 2. The distribution of the cell lines in the dendrogram were then correlated to the IC_{50} values for L-alanosine which had not been included into the cluster calculation. The median $\log_{10}$ IC_{50} value for L-alanosine (−5.0335 M) was used as a cut-off threshold to define cell lines as being sensitive or resistant. As can be seen in Table 3, cluster 1 contained

Table 1
Correlation of the constitutive mRNA expression of genes to $\log_{10}$ IC_{50} values for L-alanosine of 60 N.C.I. cell lines

Gene	Genbank accession	Name	Correlation to L-alanosine	
			P-value	COMPARE coefficient
MTHFD2	N98720	Methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	0.000046[a]	0.471[b]
	R10715	ESTs	0.000001	0.545
DKFZP434B205	W86624	Hypothetical protein similar to mouse *Fbw5*	0.000147	0.485
SNAP-29	R67087	Synaptosomal-associated protein, 29 kDa	0.000106	0.462
	T96832	Unknown	0.000053	0.454
EVI5	H51372	Ecotropic viral integration site 5	0.000032	0.452
BAT5	W48839	HLA-B associated transcript	0.000118	0.431
	H91630	Unknown	0.000082	0.431
	N93544	ESTs	0.000140	0.422
CDSN	W95595	Corneodesmosin	0.000014	0.406
	R56272	Unknown	0.000184	0.402

[a] *P*-value (Kendall's τ-test).
[b] COMPARE coefficient (Pearson *R*-value).

Table 2
Mean values (±SEM) of the mRNA expression of 11 genes or ESTs according to the two clusters shown in Fig. 2

Gene	Cluster 1		Cluster 2	
	Mean	SEM	Mean	SEM
MTHFD2	−0.0476	0.0060	0.0398	0.0071
ESTs	0.1100	0.0079	−0.1592	0.0182
DKFZP434B205	0.2104	0.0122	−0.1057	0.0139
SNAP-29	0.0815	0.0059	−0.0700	0.0056
Unknown	−0.0014	−0.0014	−0.1090	0.0054
EVI5	0.1102	0.0057	0.0208	0.0045
BAT5	0.1165	0.0035	−0.0719	0.0041
Unknown	0.1184	0.0061	−0.0529	0.0067
ESTs	0.0402	0.0040	−0.1105	0.0047
CDSN	0.0703	0.0032	−0.1295	0.0134
Unknown	0.0151	0.0056	−0.2321	0.0087

Table 3
Separation of clusters of 60 N.C.I. cell lines obtained by hierarchical cluster analysis shown in Fig. 2 in comparison to sensitivity to L-alanosine

Cluster	Sensitive (<−5.0335 M)	Resistant (>−5.0335 M)	*P*-value (Fisher's exact test)
Cluster 1	7	27	
Cluster 2	23	2	2.787×10^{-8}

The median $\log_{10} IC_{50}$ value (−5.0335 M) was used as cut-off to separate tumor cell lines as being "sensitive" or "resistant".

more resistant than sensitive tumor cell lines. *Vice versa*, cluster 2 contained more sensitive ones. This correlation was significant ($P = 2.78 \times 10^{-8}$; Fisher's exact test).

3.3. Multidrug resistance

The results of the COMPARE and FDR analyses did not indicate a role for MDR genes in resistance to L-alanosine. To investigate this possibility more thoroughly, we tested MDR cell lines that selectively overexpress either the MDR-conferring genes *MDR1*, *MRP1*, or *BCRP* [35]. Based on the ratio between the IC_{50} values of the corresponding sensitive and resistant cells, the *MDR1*-overexpressing CEM/ADR5000 cells revealed a 833-fold resistance to doxorubicin as compared to parental CCRF-CEM cells. Doxorubicin resistance of *MRP1*-overexpressing HL-60/AR cells was 149-fold and that of *BCRP*-overexpressing MDA-MB-231-BCRP clone 23 cells was 10-fold compared to the corresponding control cells. The corresponding dose–response curves for L-alanosine are given in Fig. 3. Neither CEM/ADR5000, nor HL-60/AR and MDA-MB-231-BCRP clone 23 cell lines were resistant to L-alanosine.

3.4. p53-mediated drug resistance

As the tumor suppressor p53 is another important factor of drug resistance, we also analyzed the possibility that the p53 pathway affects the response of tumor cells to L-alanosine. Comparing the p53 mutational status of the 60 N.C.I. cell lines (http://dtp.nci.nih.gov/mtargets/mt_index.html) to the IC_{50} values for L-alanosine showed a significant correlation (Fig. 4a). L-Alanosine was more active in cell lines without a p53 mutation than in p53-mutated cell lines ($P = 0.01733$; Mann–Whitney's *U*-test). As a functional measure of the p53 pathway the mRNA induction of the p53 downstream genes $p21^{WAF1/CIP1}$, *GADD45*, and *MDM2* after irradiation with γ-rays have been measured previously in the N.C.I. cell line panel [20]. In the present investigation, we correlated the induction of these genes to the IC_{50} values for L-alanosine. As can be seen in Fig. 4b–d, the degree of induction of *GADD45* and *MDM2* correlated inversely to IC_{50} values for L-alanosine ($P = 0.00314$ and 0.02236, respectively), whereas there was a trend for correlation of $p21^{WAF1/CIP1}$ to L-alanosine ($P = 0.07291$). Similarly, the induction of G1 cell cycle arrest after ionizing irradiation was compared to the sensitivity of the cell lines towards L-alanosine. Again, we observed a significant inverse correlation (Fig. 4e) that corroborates the results of Fig. 4a–d ($P = 0.01868$).

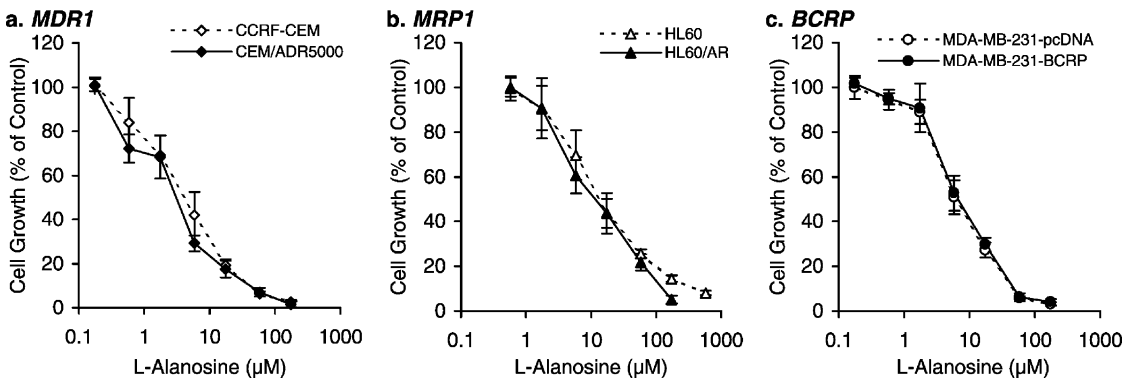

Fig. 3. Growth inhibition of *MDR1*-overexpressing CEM/ADR5000 cells, *MRP1*-overexpressing HL-60/AR cells, *BCRP*-overexpressing MDA-MB-231-BCRP clone 23 cells, and their drug-sensitive counterparts treated with L-alanosine. Controls (100%) for each cell type represent cell growth without drug addition (mean ± SD of eight determinations).

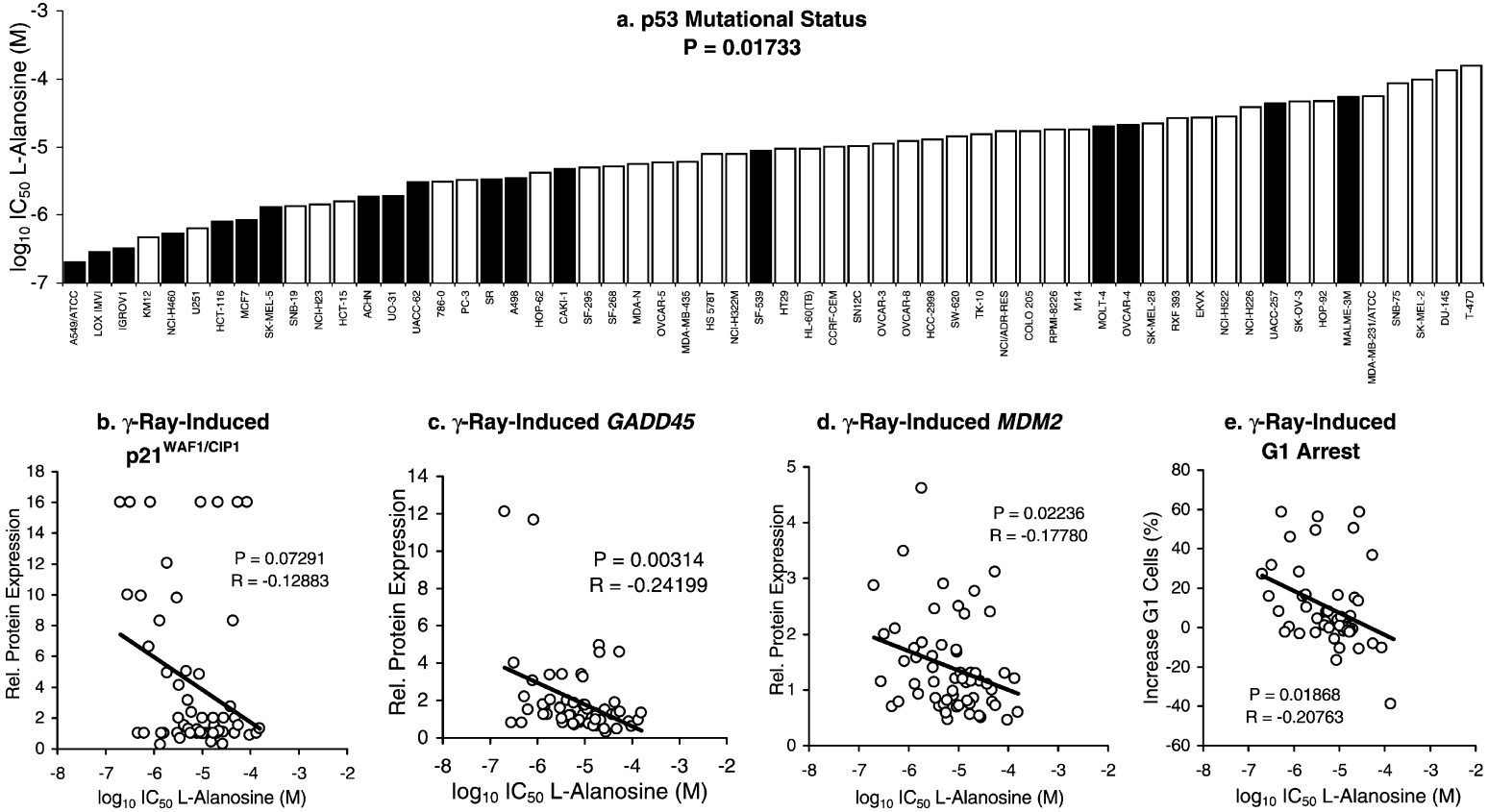

Fig. 4. Correlation of IC_{50} values for L-alanosine to the tumor suppressor gene p53, induction of p53 downstream genes, and induction of G1 arrest. (a) Ranked order of $\log_{10}$ IC_{50} values for L-alanosine of 60 cell lines of the N.C.I. in comparison to the mutational status of the tumor suppressor gene p53 ((■) wild-type p53 and (□) mutated p53). Significance level was calculated using Mann–Whitney's *U*-test. (b) Induced protein expression of (b) $p21^{WAF1/CIP1}$; (c) *GADD45*, and (d) *MDM2* after exposure to 20 Gy of γ-rays and 4 hr post-incubation. (e) Induction of G1 arrest 17 hr after exposure to 6.3 Gy. Significance levels (*P*-values) and correlation coefficients (*R*-values) were calculated using Kendall's τ-test. The degree of induction of the three genes and of G1 arrest was related to baseline protein expression or cell cycle values, respectively, as described [20].

4. Discussion

The antitumor activity of L-alanosine was first reported four decades ago [14]. The concept of a chemoselective treatment with L-alanosine raised the attraction for this drug in recent years [3,9]. Recently, we showed that methotrexate-resistant CCRF-CEM leukemia cells with a 9p21 chromosomal deletion and dihydrofolate deductase (*DHFR*) gene amplification are not cross-resistant to L-alanosine [36]. As a part of our ongoing studies, we have now analyzed the mRNA expression profiles with regard to the response of tumor cells to L-alanosine in more detail. We found that the response of 60 cell lines of the N.C.I. to L-alanosine was associated with proliferation. The IC_{50} values of L-alanosine were significantly correlated with the cell doubling times. Rapidly growing cells are more vulnerable to L-alanosine than slowly growing ones. Hence, L-alanosine's activity is at least in part dependent on the proliferative activity of tumor cells. Proliferation is an important determinant for the response of tumor cells to many antineoplastic agents [37,38].

COMPARE analyses of 9706 genes showed that the constitutive mRNA expression of 11 genes or ESTs correlated with the cytotoxic effects of L-alanosine with an error probability of 0.1%. The expression of one of these genes correlated with sensitivity to L-alanosine, while the expression of the other 10 genes correlated with drug resistance. Despite this lower statistical error probability, it is still not deducible from this analysis, whether the correlation of L-alanosine sensitivity to the activity of these genes reflects causative relations or simply epiphenomena. This type of analysis identifies candidate genes which are more likely causally related to response to L-alanosine than other genes. The validation of genes which are mechanistically relevant for cellular response to L-alanosine deserves thorough investigations in future.

The significant correlation of *MTHFD2* mRNA expression to the IC_{50} values for L-alanosine in 60 cell lines of the N.C.I. points to a possible role of *MTHFD2* for L-alanosine sensitivity. *MTHFD2* is a bifunctional enzyme of the *de novo* purine pathway catalyzing the interconversion between 5,10-methylene tetrahydrofolate and 10-formyl tetrahydrofolate. Tetrahydrofolate is crucial for the production of monocarbon units that are necessary for biosynthetic processes, including biosynthesis of purine bases, methionine, and thymidylate [39]. The *de novo* purine biosynthesis pathway results in the production of inosine monophosphate. The metabolite of L-alanosine, L-alanosyl-5-amino-4-imidazole carboxylic acid ribonucleotide is an inhibitor of adenylosuccinate synthase that converts inosinate to AMP [40]. The amount of inosinate produced *via* the *de novo* purine pathway may be linked to the cytotoxicity of L-alanosine. Hence, it can be speculated that the expression and activity of *MTHFD2* as a part of the *de novo* purine pathway may also influence L-alanosine's cytotoxic action.

Whether the other genes identified by COMPARE and FDR analyses are causatively linked to resistance to L-alanosine is unknown. *DKFZP434B205* is a hypothetical protein similar to mouse *Fbw5*. The gene has not yet an official gene symbol and name. Its interim gene symbol is *FBXW5* that stands for "likely orthologue of mouse-f-box and WD-40 domain protein 5" (http://cgap.nci.nih.gov/Genes/GeneInfo?ORG=Hs&CID=82023). *SNAP-29* participates in intracellular membrane transport processes *via* its interaction with multiple syntaxins at discrete membrane compartments [41]. The mouse *EVI5* gene and its human homologue *NB4S* share strong homology over a 200-amino acid region with the TBC1 box motif genes involved in cell growth and differentiation [42]. The *NB4S* gene is subject to t(1;10)(p22;q21) translocations fusing *NB4S* to the *TRNG10* gene. This chromosomal rearrangement creates a novel fusion gene that combines the TBC1 motif of *NB4S* with a polyadenylation signal from *TRNG10* with potentially oncogenic properties. The *BAT5* gene belongs to a cluster of HLA-B-associated genes that are localized in vicinity to the *TNFα*, *TNFβ*, and *HSP70* genes [43]. Though the functional properties of *BAT5* are unknown, the gene may play a role in immunity. Interestingly, the genetic locus within *BAT5* and the neighbored *H2D* gene is responsible for susceptibility and resistance to irradiation with ultraviolet B light [44]. Corneodesmosin is a protein in corneodesmosomes that are involved in the shedding of superficial corneocytes from the skin surface [45]. The *CDSN* gene coding for corneodesmosin has been associated to the impaired desquamation characteristic of psoriasis [46].

Correlating the IC_{50} values for L-alanosine to genes of the p53 pathway corroborated a putative role of p53 for resistance to L-alanosine. As a measure of the functional status of the p53 pathway, the induction of the p53-dependent genes $p21^{WAF1/CIP1}$, *GADD45*, and *MDM2* and of the G1 cell cycle arrest has recently been determined [20]. In the present investigation, we correlated γ-ray induced gene expression and G1 arrest to the IC_{50} values for L-alanosine under the assumption that proficiency to activate the pathway after irradiation may also occur after drug exposure. Indeed, we found inverse correlations that support this view. Our results are in good accordance with findings that connect p53 and the p53 pathway to drug resistance [20]. Hence, the present results broaden the concept of p53-mediated chemoresistance to include L-alanosine.

It is interesting that cell lines overexpressing the MDR-conferring genes *MDR1*, *MRP1*, and *BCRP*, respectively, were not cross-resistant to L-alanosine. Thus, L-alanosine is not involved in MDR phenotypes. This finding is of particular interest, since many anticancer agents of natural origin are substrates of MDR transporters [22]. This raises the hope that L-alanosine which is an antibiotic from *S. alanosinicus* may be suited to treat refractory, multidrug-resistant tumors in the clinic.

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