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Molecular Modes of Action of Artesunate in Tumor Cell Lines

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ABSTRACT

A profound cytotoxic action of the antimalarial, artesunate (ART), was identified against 55 cancer cell lines of the U.S. National Cancer Institute (NCI). The 50% inhibition concentrations (IC_{50} values) for ART correlated significantly to the cell doubling times ($P = 0.00132$) and the portion of cells in the G_0/G_1 ($P = 0.02244$) or S cell cycle phases ($P = 0.03567$). We selected mRNA expression data of 465 genes obtained by microarray hybridization from the NCI data base. These genes belong to different biological categories (drug resistance genes, DNA damage response and repair genes, oncogenes and tumor suppressor genes, apoptosis-regulating genes, proliferation-associated genes, and cytokines and cytokine-associated genes). The constitutive expression of 54 of 465 (=12%) genes correlated significantly to the IC_{50} values for ART. Hierarchical cluster analysis of these 12 genes allowed the differentiation of clusters with ART-sensitive or ART-resistant cell lines ($P = 0.00017$). For exemplary validation, cell lines transduced with 3 of the 12 genes were used to prove a causative relationship. The cDNAs for a deletion-mutated epidermal growth factor receptor (*EGFR*) and for γ-glutamylcysteine synthetase increased resistance to ART. The conditional expression of the *CDC25A* gene using a tetracycline repressor expression vector increased sensitivity toward ART. Multidrug-resistant cells differentially expressing the *MDR1*, *MRP1*, or *BCRP* genes were not cross-resistant to ART. ART acts via p53-dependent and-independent pathways in isogenic p53+/+ $p21^{WAF1/CIP1}$+/+, p53−/− $p21^{WAF1/CIP1}$+/+, and p53+/+ $p21^{WAF1/CIP1}$−/− colon carcinoma cells.

In the past decades, numerous novel synthetic and natural compounds with anticancer activity have been identified and characterized. Drugs of natural origin are indispensable in many treatment protocols (i.e., anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxanes, and others). Synthetic derivatives of natural lead compounds, e.g., idarubicin or vinorelbine, are examples of drugs with improved pharmacological features. Artesunate (ART) is a semisynthetic derivative of artemisinin, the active principle of *Artemisia annua* L. ART and other artemisinin derivatives are promising novel drugs in the treatment of malaria (Price, 2000). They are recommended by the World Health Organization as salvage treatment options for otherwise unresponsive *Plasmodium falciparum* and *Plasmodium vivax* strains. Large clinical studies with malaria patients have shown that ART is well tolerated, with few and insignificant side effects (Hien et al., 1992). In addition to the well known antimalarial activity of ART, we have previously identified a profound cytotoxic action of ART against cancer cell lines of different tumor types (Efferth et al., 2001).

Although progress in understanding of the antimalarial mechanism of artemisinin has been made (Walker et al., 2000), the molecular action of ART toward tumor cells is still unexplored. The aim of the present investigation was to analyze the modes of ART's anticancer action. In collaboration with the U.S. National Cancer Institute (NCI; Bethesda, MD), ART has been tested in 55 tumor cell lines (Efferth et

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ABBREVIATIONS: ART, artesunate; NCI, National Cancer Institute; MDR, multidrug-resistant; MRP, multidrug resistance-related protein; BCRP, breast cancer resistance protein; MSV, Moloney sarcoma virus; Tet, tetracycline; BrdU, bromodeoxyuridine; FDR, false discovery rate; GLCLR, glutamate-cysteine ligase, regulatory (30.8 kDa), γ-glutamylcysteine synthetase; EGFR, epidermal growth factor receptor; RT-PCR, reverse transcribed-polymerase chain reaction; ABC, ATP-binding cassette.

al., 2001). Now, we have analyzed these data, together with the mRNA expression measured by microarray hybridization (Scherf et al., 2000) and with cell cycle and proliferation parameters (O'Connor et al., 1997) by Kendall's τ test and hierarchical cluster analysis. For target validation, cell lines transduced with genes identified by cluster analysis were taken as examples to prove a causative role of these genes for the response to ART. Furthermore, we analyzed the activity of ART against multidrug-resistant (MDR) tumor cells that differentially expressed the MDR-conferring *MDR1*, multidrug resistance-related protein 1 (*MRP1*), or *BCRP* genes.

Materials and Methods

Drugs

ART was obtained from Saokim Co. Ltd. (Hanoi, Vietnam).

Cells and Cell Lines.

Cell Lines of the Developmental Therapeutics Program of the NCI The panel for the present investigations consisted of 55 human tumor cell lines representing leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian carcinoma, tumors of the central nervous system, prostate carcinoma, and breast cancer. The cell lines and their cultivation have been described previously (Alley et al., 1988). Cells were assayed by means of a sulforhodamine B assay (Rubinstein et al., 1990).

Transduced Cell Lines. MSV-HL13 cells transduced with pcDNA3 expression vectors harboring cDNAs for heavy and light subunits of γ-glutamylcysteine synthetase and MSV-PC4 cells carrying a pcDNA3 mock control expression vector were handled as described (O'Brien et al., 2000). MSV cells are a clonal variant of NIH3T3 cells. The establishment of human glioblastoma multiforme U-87MG cell lines transduced with a mock control expression vector (U-87MG.Lux) or an expression vector harboring an epidermal growth factor receptor (*EGFR*) gene with a genomic deletion of exons 2 through 7 (U-87MG.ΔEGFR) has been reported previously (Huang et al., 1997). The 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). Rat embryo R12 cells containing the tetracycline (Tet) repression expression vector pUHD 15-1 and a hygromycin resistance vector were previously described. *CDC25A* expression was inducible after tetracycline removal for 48 h, resulting in a 5-fold increase in CDC25A phosphatase activity (Blomberg and Hoffmann, 1999).

Multidrug-Resistant Tumor Cell Lines. Leukemic CCRF-CEM and HL-60 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 with cells in the logarithmic growth phase. The multidrug resistance gene 1 (*MDR1*)-expressing CEM/ADR5000 was maintained in 5000 ng/ml doxorubicin. The *MRP1*-expressing HL-60/AR subline was continuously treated with 100 nM daunorubicin. The establishment of the resistant subline has been 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 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 HL-60 cells. The 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).

Knockout Cell Lines. Human wild-type HCT116 colon cancer cells (p53+/+ $p21^{WAF1/CIP1}$+/+) as well as knockout clones (p53−/− $p21^{WAF1/CIP1}$+/+, and p53+/+ $p21^{WAF1/CIP1}$−/−) derived by homologous recombination (Waldman et al., 1995; Bunz et al., 1998; Weber et al., 2002) 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.

Growth Inhibition Assay

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

Reverse Transcribed-Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted by using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) and the RNase-free DNase set (QIAGEN), and dissolved in diethylpyrocarbonate-treated H_2O. Two micrograms of RNA were reverse-transcribed using 20 pmol of oligo(dT) primer and the Omniscript reverse transcriptase kit (QIAGEN) in a final volume of 40 μl. The reverse transcription mixture (50 μl) was diluted with the same volume of H_2O, and 2 μl (100 ng) of cDNA was added to 48 μl of PCR mix. The PCR mix contained 5 μl of 10× reaction buffer, 5 μl of deoxynucleoside-5′-triphosphate mix (2 mM for each deoxynucleoside-5′-triphosphate), 5 μl of each primer (5 pmol/μl), 0.25 μl (5 U/μl) *Taq* Polymerase (Roche Diagnostics, Mannheim, Germany), and 27.75 μl of H_2O. A 5-min denaturation step at 94°C was followed by 29 cycles (*MDR1*), 26 cycles (*MRP1*), and 24 cycles (*BCRP*) of amplification, respectively. Each cycle was composed of denaturation (94°C, 30 s), primer annealing (55°C, 30 s), and primer extension (72°C, 30 s). The PCR was completed by a final extension step (72°C, 10 min). The cycle number for each gene was determined by estimation of the linear region of gene amplification by subjecting a cDNA mix to an increasing number of PCR cycles. The negative controls were constructed by using the cDNA synthesis mixture as described above, without addition of reverse transcriptase or containing water instead of cDNA. PCR products were electrophoretically separated onto 2.0% agarose gels and stained with ethidium bromide.

The primers were: *MDR1 (ABCB1)*, 1524 5′-ATT GGT GTG GTG AGT CAG GAA-3′ 1544 and 1908 5′-TGA CGT CAG CAT TAC GAA CT-3′ 1889; *MRP1 (ABCC1)*, 2296 5′-CGT GTA CTC CAA CGC TGA C-3′ 2314 and 2621 5′-CTG GAC CGC TGA CGC CCG TG-3′ 2602; *BCRP (ABCG2)*, 1025 5′-AGA CTT ATG TTC CAC GGG CC-3′ 1044 and 2138 5′-CCA AGG CCA CGT GAT TCT TC-3′ 2119.

Immunoblotting and Kinase Assays

The procedures have been described (Funk et al., 1997). Briefly, whole-cell lysates were prepared by brief sonication in lysis buffer containing 50 mM HEPES, pH 7.5, 450 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 0.1% Tween 20. For immunoblotting, 20 μg of whole-cell extract were loaded. Antibodies were sourced as follows: anti-p53 and anti-p21 (Oncogene Science, Cambridge MA), anti-RB (BD Biosciences PharMingen, Heidelberg, Germany), anti-CDK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-cyclin B1 (GNS-1; BD Biosciences PharMingen), and anti-CDC25A (Santa Cruz Biotechnology). In vitro kinase assays were performed as described previously in detail (Hermeking et al., 1995). For immunoprecipitation, antibodies against CDK2 or cyclin B1 were used.

Flow Cytometry

Cell samples were fixed in 70% ethanol in phosphate-buffered saline after labeling with bromodeoxyuridine (BrdU; Sigma Chemie,

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Deisenhofen, Germany), stained with 50 μg/ml propidium iodide (Sigma Chemie), and subjected to flow cytometry using a Becton Dickinson FacScan II (BD Biosciences).

Statistical Analyses

Objects were classified by calculation of distances according to the closeness of between-individual distances by means of hierarchical cluster analysis. All objects were 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 distance of a subordinate cluster 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, whereas the separation in the cluster tree increases with progressive dissimilarity. Recently, cluster models have been applied for gene expression profiling and for approaching molecular pharmacology of cancer (Efferth et al., 1997; Scherf et al., 2000; Volm et al., 2002). Cluster analyses applying the complete-linkage method were done with the WinSTAT program (Kalmia Company, Inc., Cambridge, MA). 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. 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.

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 dependence 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 and metric scaling of data are 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.

The Mann-Whitney U test is an implement of the WinSTAT program (Kalmia). The test was used to analyze two rows of values for significant differences. It is a parameter-free test without need for normal distribution. The χ^2 test (WinSTAT, Kalmia) was applied to bivariate frequency distributions of pairs of nominal scaled variables.

COMPARE analyses were performed with software implemented into the web site of the NCI (http://dtp.nci.nih.gov). COMPARE analyses yielded rank-ordered lists of compounds. Every compound of the Standard Agent Database of the NCI was ranked for similarity between its modulation of in vitro cell growth patterns and the modulation of in vitro cell growth patterns of a selected seed or probe compound (Paull et al., 1989). To obtain COMPARE rankings, a scale index of similarity between the seed compound cell growth pattern and the pattern for each of the COMPARE data base compounds is created. This methodology is used to identify the presumable mode of action of investigational drugs by comparing their IC_{50} profiles using the NCI cell lines with those of drugs with well characterized mechanisms of action.

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 (Hochberg and Benjamini, 1990; Keselman et al., 2002). Therefore, a step-up resampling multicomparison procedure was applied to control the false discovery rate (FDR) among the significant correlations at significance levels of 0.05. This program has been developed by Reiner et al. (2003) and 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 (Reiner et al., 2003).

Results

COMPARE Analysis. A concentration range from 10^{-8} to 10^{-4} M ART was previously tested in 55 cell lines of the Developmental Therapeutics Program of the U.S. NCI (Efferth et al., 2001). The IC_{50} values were in a range from 246 nM to ≥100 μM ($\log_{10}$, −6.609 to ≥ −4). These data were subjected to COMPARE analysis. The COMPARE computations of IC_{50} values for ART against 171 agents included in the Standard Agent Database of the NCI did not reveal any information regarding the mode of action of ART (data not shown). Thus, ART does not seem to belong to any traditional class of antitumor drugs (e.g., topoisomerase I/II inhibitors, tubulin poisons, DNA/RNA inhibitors, etc.). Therefore, we searched for other cellular and molecular determinants of ART's action against tumor cells.

Proliferative Activity and Cell Cycle Distribution. A large number of cellular and molecular parameters have been analyzed in the cell lines of the NCI's screening panel (http://dtp.nci.nih.gov) (O'Connor et al., 1997; Scherf et al., 2000). We first correlated IC_{50} values of ART to various proliferation parameters. As shown in Fig. 1a, there was a significant positive correlation between cell doubling times of untreated cells and IC_{50} values for ART (P = 0.00132; R = 0.27879). Thus, rapidly growing cell lines were more susceptible to ART than slowly growing ones. Next, the cell cycle distribution of the 55 cell lines in relation to ART's activity was analyzed. We observed a positive correlation between IC_{50} values of ART and percentage of cells in the G_0/G_1 phase of the cell cycle (P = 0.02244; R = 0.20232) and an inverse correlation between ART's IC_{50} values and S-phase proportions of the cell lines (P = 0.03567; R = −0.18190). Cell lines with a low percentage of G_0/G_1 cells and a high percentage of S-phase cells had a high proliferative activity and were most sensitive to ART. G_2/M phases did not significantly correlate with the inhibitory action of ART (Fig. 1d)

For comparison, the relationship between proliferation and growth-inhibiting activity was also analyzed for standard anticancer agents. Table 1 shows the P values obtained by Kendall's τ test. The IC_{50} values for doxorubicin, vinblastine, 5-fluorouracil, and methotrexate correlated significantly with the doubling times of the cell lines of the NCI panel, whereas those for carboplatin, dacarbazine, and ifosfamide did not. If the cell cycle distribution was compared with the IC_{50} values, the S-phase fractions correlated significantly with the IC_{50} values for 5-fluorouracil and methotrexate. The cell fractions in G_2/M phase were significantly associated with the IC_{50} values for doxorubicin, etoposide, vinblastine, 5-fluorouracil, and methotrexate. The G_0/G_1 phases did not correlate with the IC_{50} values for the drugs analyzed. Furthermore, carboplatin, dacarbazine, and ifosfamide did not correlate to any cell cycle phase.

mRNA Expression Profiling. IC_{50} values of ART were then correlated with the constitutive mRNA expression levels measured by microarray hybridization. We selected expression data of 465 genes deposited in the NCI's data base (http://dtp.nci.nih.gov). These genes belong to six categories of different biological functions (drug resistance genes, DNA damage response and repair genes, oncogenes and tumor suppressor genes, apoptosis-regulating genes, proliferation-associated genes, and cytokines and cytokine-associated genes). These categories of genes were chosen because they are frequently involved in the response of tumor cells to established antineoplastic drugs. For this reason, the data base of the Developmental Therapeutics Program of the NCI was searched for several text strings (resistance, damage,

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repair, oncogene, tumor suppressor, proliferation, cell cycle, growth, mitosis, apoptosis, death, cytokine, interferon, interleukin). Furthermore, genes were included in the analysis that are well known from the literature to affect chemosensitivity and chemoresistance. After correlation analysis by means of Kendall's τ test, the significance level was adjusted to 0.05 for FDR calculation. The resulting α-value of 0.00573 indicated that only 54 of 465 genes (12%) correlated significantly to ART response, with an error probability of $\leq 5\%$ (Table 2).

The baseline mRNA expression data were subjected to hierarchical cluster analyses (Fig. 2). The dendrogram of the 55 cell lines was divided into three main clusters. To examine whether these clusters were associated with the response to ART, these clusters were correlated to the IC_{50} data for ART that had not been included before the cluster analysis. The

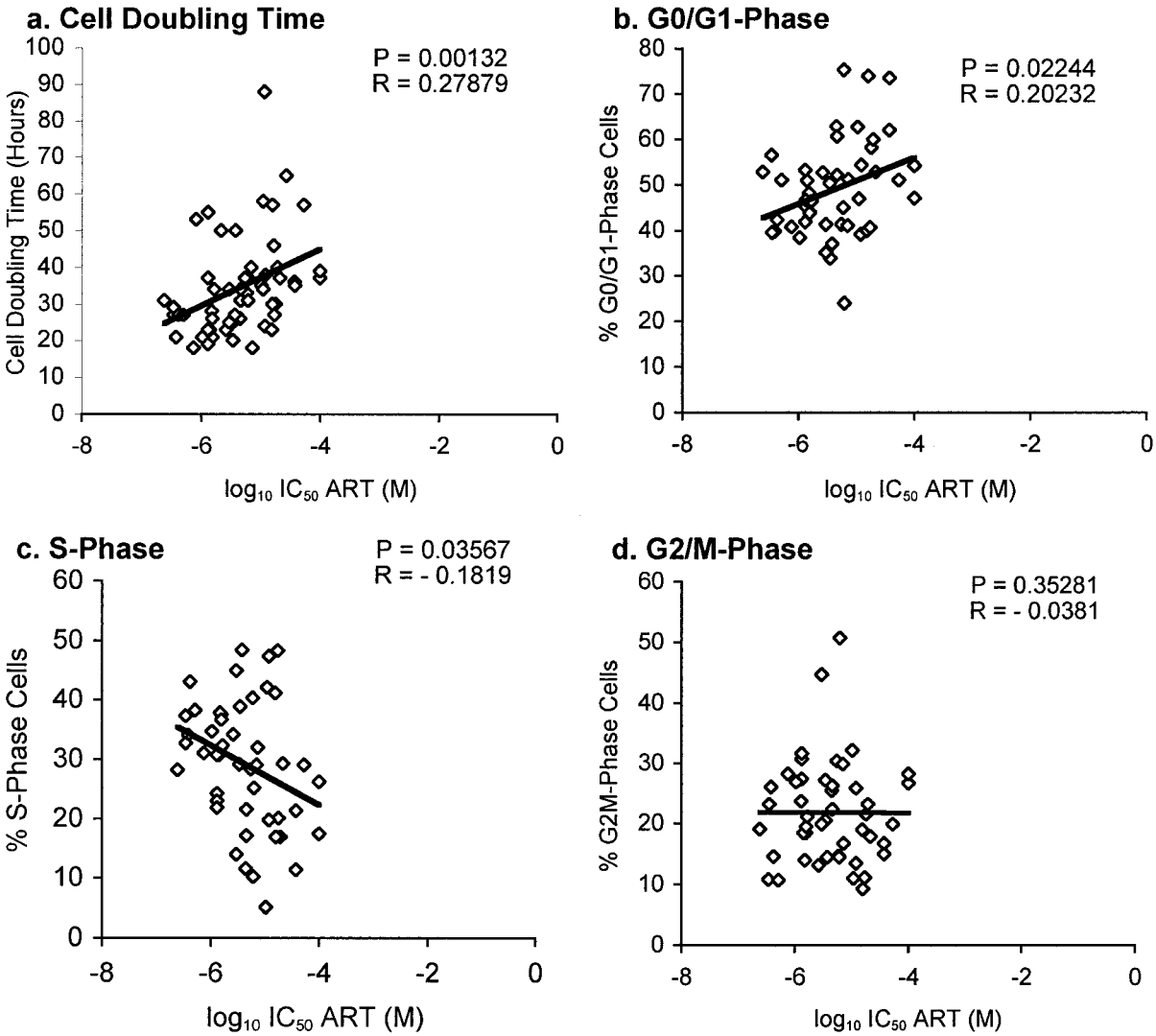

Fig. 1. Linear regression of $\log_{10}$ IC_{50} values for ART of cell lines included in the screening panel of the Developmental Therapeutics Program (NCI) in comparison to the cell doubling time (a), the G_0/G_1-phase fractions of the cell cycle (b), the S-phase fractions (c), and the G_2/M-phase fractions (d). Significance levels were calculated using Kendall's τ test.

TABLE 1

Correlation of proliferation parameters (cell doubling time and cell cycle phases) with the IC_{50} values for standard antineoplastic agents in the NCI cell line panel

Numbers are *P* values (Kendall's τ test).

	Doubling Time	G_0/G_1	S	G_2/M
Doxorubicin	0.01445	0.15533	0.17845	0.00371
Vinblastine	0.00248	0.46660	0.06903	0.01866
5-Fluorouracil	<0.00001	0.46994	0.01889	0.06871
Methotrexate	<0.00001	0.13427	0.00505	0.00936
Carboplatin	0.47414	0.14364	0.22549	0.11834
Dacarbazine	0.33905	0.32246	0.35002	0.14325
Ifosfamide	0.34686	0.41963	0.23635	0.38028

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median IC_{50} value of all 55 cell lines tested ($log_{10} = -5.335$ M) was used as the cut-off threshold to separate sensitive from resistant cell lines. Interestingly, cluster 1 ($n = 6$) and cluster 2 ($n = 25$) contained significantly more ART-sensitive tumor cell lines, whereas cluster 3 ($n = 24$) was enriched with ART-resistant ones (Table 3). As calculated by means of the χ^2 test, the distribution of sensitive and resistant tumor cell lines in these clusters is significantly different ($P = 0.00017$;

TABLE 2
Correlation of baseline mRNA expressions of genes to IC_{50} values of ART in 55 cell lines of the NCI

Symbol	Name	Correlation to IC_{50} of ART[a]	
		P Value	*R* Value[b]
Drug Resistance Genes			
GLCLR	Glutamate-cysteine ligase, regulatory (30.8 kD), γ-glutamylcysteine synthetase	0.00051	0.30452
GSTT2	Glutathione *S*-transferase T2	0.00136	0.27787
MGST3	Microsomal glutathione *S*-transferase 3	0.00246	0.27144
TOP1	DNA topoisomerase I	0.00001	−0.38821
=4/63 genes (=6%)			
DNA Damage and Repair Genes			
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5	0.00074	−0.29471
UNG	Uracil-DNA glycosylase	0.00116	−0.28233
FEN1	Flap structure-specific endonuclease 1	0.00014	−0.33695
LIG1	ATP-dependent DNA ligase I	0.00037	−0.31290
RPS3	Ribosomal protein S3	<0.00001	−0.41150
UBE2A	Ubiquitin-conjugating enzyme E2A; *RAD6* homolog	0.00236	0.26201
HMG1	High-mobility group 1	0.00049	−0.30555
HMG17	High-mobility group 17	0.00085	0.29403
=8/54 genes (=15%)			
Oncogenes and Tumor Suppressor Genes			
BRCA2	Breast cancer susceptibility gene 2	0.00094	−0.28832
EGFR	Epidermal growth factor receptor	0.00390	0.24678
EMP1	Epithelial membrane protein 1	0.00457	0.24412
FOSL2	*FOS*-like antigen-2	0.00166	0.27229
IRF4	Interferon regulatory factor 4	0.00156	−0.27402
MEN1	Multiple endocrine neoplasia 1	0.00278	−0.25719
MYB	*v-myb* avian myeloblastosis viral oncogene homolog	0.00006	−0.36127
MYC	*v-myc* avian myelocytomatosis viral oncogene homolog	0.00002	−0.38498
CSK	*c-src* tyrosine kinase	0.00142	−0.27672
TGFB2	Tumor growth factor β2	0.00345	0.25816
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	0.00456	0.24187
BRAF	*v-raf* murine sarcoma viral oncogene homolog B1	0.00031	−0.32033
RAB2	Member *RAS* oncogene family 2	0.00047	0.30686
RAN	Member *RAS* oncogene family	0.00315	−0.25330
ARHC	*RAS* oncogene family member C	0.00004	0.36634
ARHE	*RAS* oncogene family member E	0.00097	0.28755
=16/112 genes (=14%)			
Apoptosis-Regulating Genes			
LOC51272	Locus 51272	0.00153	−0.27457
CIDEB	Cell death-inducing *DFFA*-like effector b	0.00022	−0.32566
PDCD2	Programmed cell death 2	0.00040	−0.31118
BAG1	*BCL2*-associated athanogene	0.00045	−0.29123
BAG3	*BCL2*-associated athanogene 3	0.00505	0.23856
MADD	*MAP*-kinase activating death domain	0.00720	−0.22917
=5/65 genes (=8%)			
Proliferation-Associated Genes			
DNL	Dynein, cytoplasmic, light polypeptide	0.00039	0.31182
KIF5B	Kinesin family member 5B	0.00005	0.36333
TUBB4	β-Tubulin 4	0.00260	0.15057
LOC57018	Cyclin L ania-6a	0.00558	−0.23537
CDK8	Cyclin-dependent kinase 8	0.00019	−0.32938
CDKN3	Cyclin-dependent kinase inhibitor 3	0.00262	−0.26151
CDC25A	Cell division cycle 25A gene	0.00055	−0.30264
CEP3	*CDC42* effector protein 3	0.00212	0.26513
SPEC1	Small protein effector 1 of *CDC42*	0.00065	0.30143
APC5	Anaphase-promoting complex subunit 5	0.00109	−0.28426
APC7	Anaphase-promoting complex subunit 7	0.00200	−0.26684
GSPT1	G_1 to S phase transition 1 gene	0.00449	−0.24230
MPHOSPH10	M-phase phosphoprotein 10	0.00259	−0.25931
MPHOSPH6	M-phase phosphoprotein 6	0.00069	−0.29662
PA2G4	Proliferation-associated *2G4* gene, 38 kD	0.00003	−0.36954
CTGF	Connective tissue growth factor	0.00138	0.27747
VEGFC	Vascular endothelial growth factor C	0.00125	0.28038
=17/123 genes (=14%)			
Cytokines			
SCYB13	Small inducible cytokine B subfamily, member 13	0.00178	0.27571
IL13RA1	Interleukin 13 receptor α 1	0.00001	0.39040
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.00131	0.27905
CSF1	Colony-stimulating factor 1	0.00001	0.39986
=4/48 genes (=8%)			
Total: 54/465 genes (= 12%)			

[a] Significance (*P* values) and correlation coefficients (*R* values) were calculated by Kendall's τ test
[b] Negative *R* values indicate that mRNA expression correlates inversely with IC_{50} values for ART; positive *R* values indicate a direct correlation of both parameters.

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Table 3). Cluster 1 contained only leukemia cell lines, whereas clusters 2 to 3 consisted only of cell lines of solid tumors.

Effect of ART on Transduced Cell Lines. To start analyzing the functional role of some of these genes in the cellular response to ART, target validation experiments were performed in selected cases by treating transduced cell lines with ART. We selected three genes from the panel of 60 that belong to three different functional groups, e.g., detoxification of radical molecules and reactive oxygen species (glutamate-cysteine ligase, regulatory (30.8 kDa), γ-glutamylcysteine synthetase, *GLCLR*), oncogenic transformation (epidermal growth factor receptor, *EGFR*), or cell cycle regulation (*CDC25A*). The three genes were chosen by the following criteria: 1) an error probability of ≤5% in FDR calculations, 2) a significance value of $P < 0.005$, and 3) the availability of suitable cell models.

In the NCI cell line panel, a significant correlation between mRNA expression of *GLCLR* and IC_{50} values for ART was found (Fig. 3a, $P = 0.00051$; $r = 0.30452$). To corroborate the relationship of ART to *GLCLR*, we treated MSV-HL13, transduced with the γ-glutamylcysteine synthetase heavy and light subunits, and MSV-PC4, transduced with mock control vector. These transduced cell lines have been previously described (O'Brien et al., 2000). The IC_{50} values calculated from the dose-response curves in Fig. 3d showed a 3.5-fold increase in drug resistance in MSV-HL13 cells compared with MSV-PC4 cells.

The *EGFR* mRNA expression of the NCI cell line panel correlated significantly with the IC_{50} values for ART (Fig. 3b; $P = 0.00390$; $R = 0.24678$). Therefore, we opted to analyze the relationship between cellular response to ART and expression of *EGFR*. U-87MG cells transduced with a truncated *EGFR* (U-87MG.ΔEGFR) or with control expression vector (U-87MG.Lux) have been reported elsewhere (Huang et al., 1997). Both cell lines were exposed to ART. The IC_{50} values calculated from the dose-response curves in Fig. 3e showed that U-87MG.ΔEGFR cells were 13.6-fold more resistant to ART than were the U-87MG.Lux cells, indicating a causative role of the *EGFR* for resistance of tumor cells to ART.

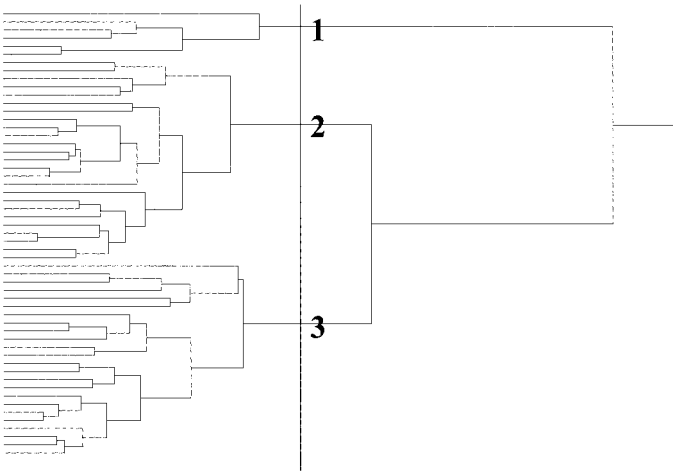

Fig. 2. Dendrogram obtained by hierarchical cluster analysis (complete linkage method). The mRNA expression data of 60 genes (see Table 2) of 55 cell lines of the NCI were included in the analysis. The IC_{50} values for ART themselves were not included in the cluster computation. The dendrogram is divided into three main clusters. Cell lines of cluster 1 and 2 were more sensitive toward ART than were cell lines of cluster 3.

An inverse correlation between *CDC25A* mRNA expression and the IC_{50} values for ART in the 55 NCI cell lines was observed (Fig. 3c; $P = 0.00055$; $R = -0.30264$). Furthermore, we used R12 cells transduced with the Tet repressor expression vector pUHD 15-1 containing *CDC25A*. The repression of *CDC25A* expression in this Tet-off model has been described (Blomberg and Hoffmann, 1999). Aliquots of R12 cells were cultured in the presence or absence of tetracycline. Removal of tetracycline for 48 h sensitized R12 cells 6.9-fold toward ART in comparison to tetracycline-pretreated cells (Fig. 3f). With this set of experiments, 3 of 60 genes identified by correlation analyses (Table 2) were validated as causally related to the cellular response toward ART.

For comparison, a parallel experimentation was set up for doxorubicin as a standard anticancer drug. A significant correlation was observed between the IC_{50} values for doxorubicin and mRNA expression of *EGFR* in the NCI cell line panel (Fig. 4b, $P = 0.01546$; $R = 0.18948$) but not for *GLCLR* or *CDC25A* (Fig. 4, a and c). Furthermore, over-expression of *EGFR* in U-87MG.ΔEGFR cells resulted in a 3.2-fold resistance to doxorubicin as compared with U-87MG.Lux control cells (Fig. 4d). The *GLCLR*-transfectant MSV-HL13 cells were 1.4-fold resistant to doxorubicin (Fig. 4e), whereas *CDC25A* did not have any influence on doxorubicin sensitivity in R12 Tet-off cells (Fig. 4f).

Comparison of ART to Established Anticancer Drugs. Next, we compared the number of genes correlating with the IC_{50} values for ART to those correlating to 15 established anticancer agents. Figure 5 shows the number of genes in the NCI cell line panel in which mRNA expression correlated to the IC_{50} values of the various anticancer drugs. Interestingly, ART was among the drugs that correlated with most genes of all gene categories except for drug resistance genes. This indicates that these gene categories may play a less important role in determining ART's inhibitory action on tumor cells than genes of the other gene groups analyzed.

Multidrug Resistance. The drug comparison analysis in Fig. 5 stimulated us to investigate the role of drug resistance in determining ART's cellular effects in more detail. For this reason, the effects of ART on different multidrug-resistant tumor cells was investigated. As shown by reverse-transcribed polymerase chain reaction (RT-PCR), these cell lines expressed individual mRNA species for MDR-conferring ATP-binding cassette (ABC) transporter genes (Fig. 6c). Doxorubicin-resistant CEM/ADR5000 cells over-expressed *MDR1* (*ABCB1*), doxorubicin-resistant HL-60/AR cells over-

TABLE 3

Separation of four clusters of tumor cell lines obtained by hierarchical cluster analysis shown in Fig. 2 in comparison to sensitivity to ART

The median IC_{50} value ($\log_{10} = -5.335$ M) was used as a cut-off to separate tumor cell lines as being "sensitive" or "resistant". $P = 0.00046$ (χ^2 test).

	Cluster 1	Cluster 2	Cluster 3	Sum
Sensitive	6	17	5	28
Resistant	0	8	19	27
Sum	6	25	24	55

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expressed the MDR-related protein 1 (*MRP1*; *ABCC1*), and MDA-MB-231-BCRP clone 23 cells over-expressed the breast cancer resistance protein (*BCRP*, *ABCG2*). The corresponding drug-sensitive cell lines did not over-express any of these ABC transporter genes. Trace amounts of *MRP1* mRNA were detected in both sensitive and resistant CEM cells. The drug resistance phenotype of these cell lines was verified with doxorubicin using growth inhibition assays (Fig. 6a). As calculated from IC_{50} values of sensitive and resistant cells, CEM/ADR5000 cells revealed 833-fold, HL-60/AR cells 149-fold, and MDA-MB-231-BCRP clone 23 cells 10-fold resistance toward doxorubicin. Interestingly, ART did not encounter cross-resistance in any of these resistant cell lines (Fig. 6b), indicating that ART is not subject to MDR mechanisms constituted by these ABC transporter genes.

p53-Mediated Chemoresistance. Since the tumor suppressor p53 is another important factor of chemoresistance, we also analyzed the possibility that p53 affects the response of tumor cells to ART. Interestingly, p53 was not among the genes identified in Table 2. Comparing the p53 mutational status of the NCI cell line panel (http://dtp.nci.nih.gov) to the IC_{50} values for ART did not show a significant correlation (Fig. 7; Mann-Whitney *U* test). Furthermore, we tested the p53-dependent downstream gene $p21^{WAF1/CIP1}$. Again, we did not find a correlation of the IC_{50} values for ART and the constitutive mRNA expression of $p21^{WAF1/CIP1}$ (data not shown).

Considering the paramount importance of the p53 pathway for drug resistance, we analyzed p53 and $p21^{WAF1/CIP1}$ in more detail. For this reason, we used human wild-type HCT-116 colon cancer cells (p53+/+ $p21^{WAF1/CIP1}$+/+) and isogenic knockout clones (p53−/− $p21^{WAF1/CIP1}$+/+ and p53+/+ $p21^{WAF1/CIP1}$−/−) derived by homologous recombination (Waldman et al., 1995; Bunz et al., 1998; Weber et al., 2002). First, we measured the incorporation of BrdU in the three cell lines without and with 10^{-5} M ART for 24 or 48 h by flow cytometry (Fig. 8a). ART inhibited the BrdU incorporation in all three cell lines in a time-dependent manner and to a similar extent. This indicates that the two knockout cell lines were similarly sensitive to ART-induced inhibition of proliferation as wild-type HCT-116 cells. Using immunoblotting and kinase assays, we analyzed the protein expression and kinase activity of cell cycle regulating genes in wild-type cells and knockout mutants (Fig. 8, b and c). Treatment with ART induced the expression of p53 protein after 12 and 24 h in wild-type cells but not in p53+/+ $p21^{WAF1/CIP1}$−/− knockout cells (Fig. 8b). The $p21^{WAF1/CIP1}$ protein was strongly induced in wild-type cells and very weakly

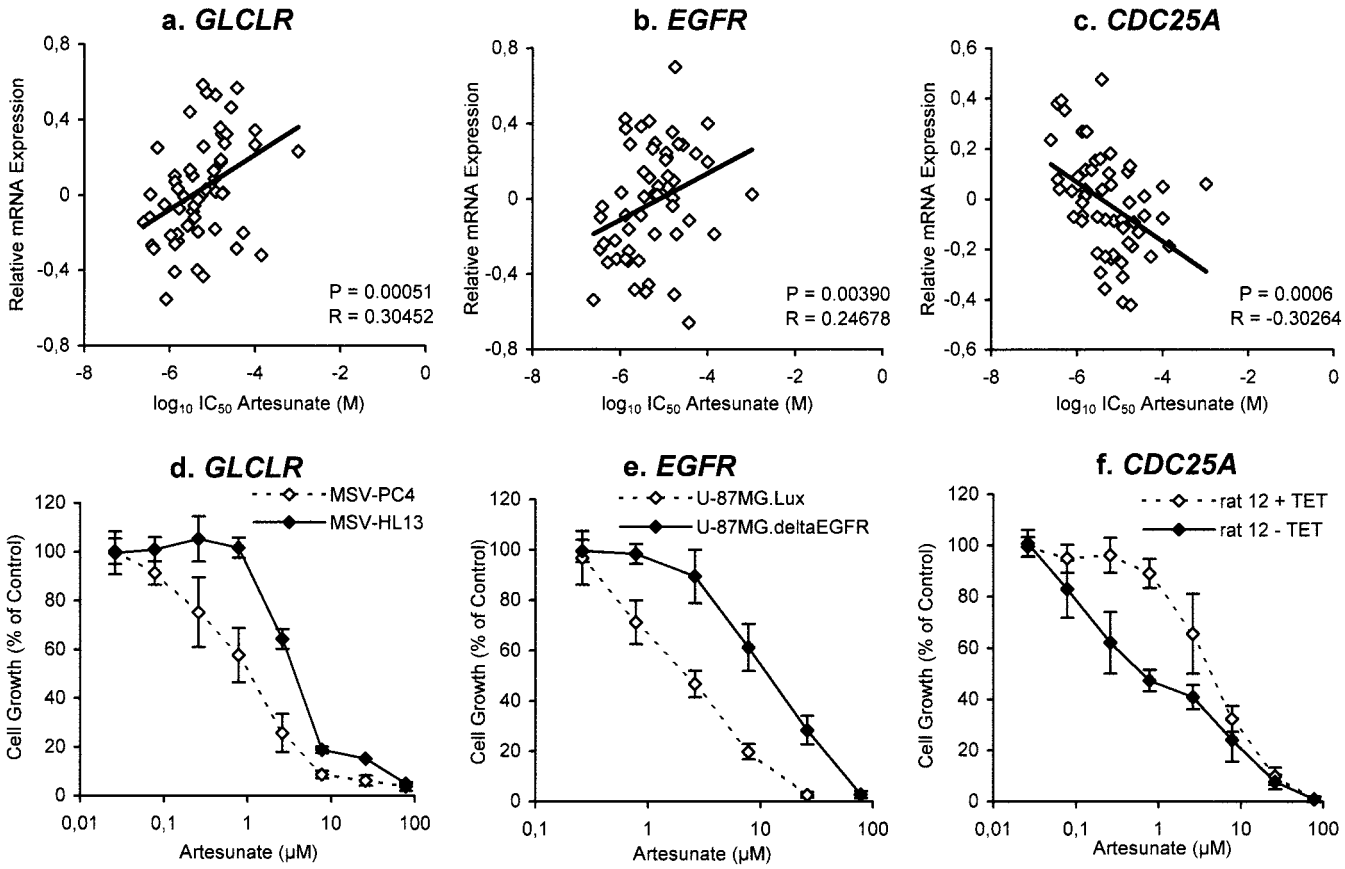

Fig. 3. Effect of ART on cell lines transduced with selected genes identified by hierarchical cluster analysis (Table 1; Fig. 2). Upper row, linear regression of $\log_{10}$ IC_{50} values for ART and mRNA expression of γ-glutamylcysteine synthetase (a), epidermal growth factor receptor (*EGFR*) (b), and *CDC25A* (c) in the NCI cell line panel. Significance level was calculated using Kendall's τ test. Lower row, growth inhibition assays of MSV cells after treatment with ART. d, MSV-PC4 cells transduced with mock control expression vector and MSV-HL13 cells transduced with γ-glutamylcysteine synthetase heavy and light subunits (mean ± S.D. of eight determinations); e, U-87MG.Lux cells transduced with mock control expression vector and U-87MG.ΔEGFR cells transduced with *EGFR* truncated at exons 2 to 7; and f, R12 cells containing tetracycline repressor vector pUHD 15-1 with a *CDC25A* gene. ART was applied to cells that were constantly maintained with tetracycline or with cells after removal of tetracycline for 48 h. Mean ± S.D. of eight determinations.

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induced in p53−/− p21$^{WAF1/CIP1}$ +/+ cells upon ART treatment. Hypophosphorylation of the tumor suppressor protein RB coincided with a down-regulation of *CDK2* kinase activity in response to ART treatment that is indicative of G_1/S arrest. Protein expression and kinase activity of the G_2/M regulator cyclin B1 declined after treatment of all three cell lines with ART for 12 and 24 h. Because *CDC25A*, which governs the entry of G_1 cells into the S phase was causally related to ART sensitivity (Fig. 3f), we also analyzed this protein in the knockout cell lines. Expression of *CDC25A* protein was down-regulated 24 and 48 h after treatment with ART (Fig. 8c).

Discussion

In the present investigation, we have analyzed molecular modes of action of the antimalarial ART on cancer cells. The IC_{50} values for ART in 55 NCI cell lines ranged from 246 nM to ≥100 μM (log_{10}, −6.609 to ≥−4). In clinical antimalaria studies, plasma peak concentrations of 2640 ± 1800 μg/ml (= 6.88 ± 4.69 mM) have been measured upon intravenous application of 2 mg/kg ART in patients (Batty et al., 1996). Since the IC_{50} values for ART of the 55 cell lines are about 3 orders of magnitude lower than these plasma levels, it is reasonable to speculate that sufficient concentrations may be achievable to inhibit tumor cell growth in human beings.

A survey of 465 genes showed that the constitutive expression of 54 genes correlated significantly with the response to ART at a FDR probability of ≤5%. This was a surprisingly high number of genes. It should, however, be considered that the correlation coefficients for these 54 genes were rather small. From this analysis it is still not clear whether the correlations reflect causative relationships or simply epiphenomena. Nevertheless, this type of analysis represents a valuable source to produce testable hypotheses.

The expression of several drug resistance genes correlated with the IC_{50} values of ART, especially of genes that are involved in the detoxification of electrophilic compounds (glutathione-associated enzymes). This is in accordance with the reaction of ART's endoperoxide bridge to form reactive oxygen species (ROS) and/or ART carbon-centered free radicals, both of which affect cellular proteins and lipids of the parasites (Asawamahasakda et al., 1994; Berman and Adams, 1997). A role for the endoperoxide bridge can also be assumed for the inhibitory activity in tumor cells. The glutathione *S*-transferase enzyme family catalyzes the conjugation of radical molecules and ROS to reduced glutathione. Cellular glutathione content is regulated by GLCLR, which is crucial for the cellular detoxification capacity of a number of xenobiotics. Because GLCLR was one of the 54 candidate genes, we analyzed its importance in determining ART cytotoxicity in more detail. Indeed, transduced cells were more resistant to ART than were control cells. Of interest, there was only a mild influence of GLCLR on doxorubicin cytotoxicity. This could imply that in the transfectant, the quinone formed from doxorubicin is not as good an electrophilic substrate as the endoperoxide bridge of artesunate.

In comparison with established anticancer drugs, ART correlated with fewer drug resistance genes. This indicates that drug resistance genes may be a less important issue if ART is established as a viable anticancer drug. This observation fits well with a recent investigation (Efferth et al., 2002a). ART was more potent than the artemisinin derivatives arteether and artemether, and the number of drug resistance genes in

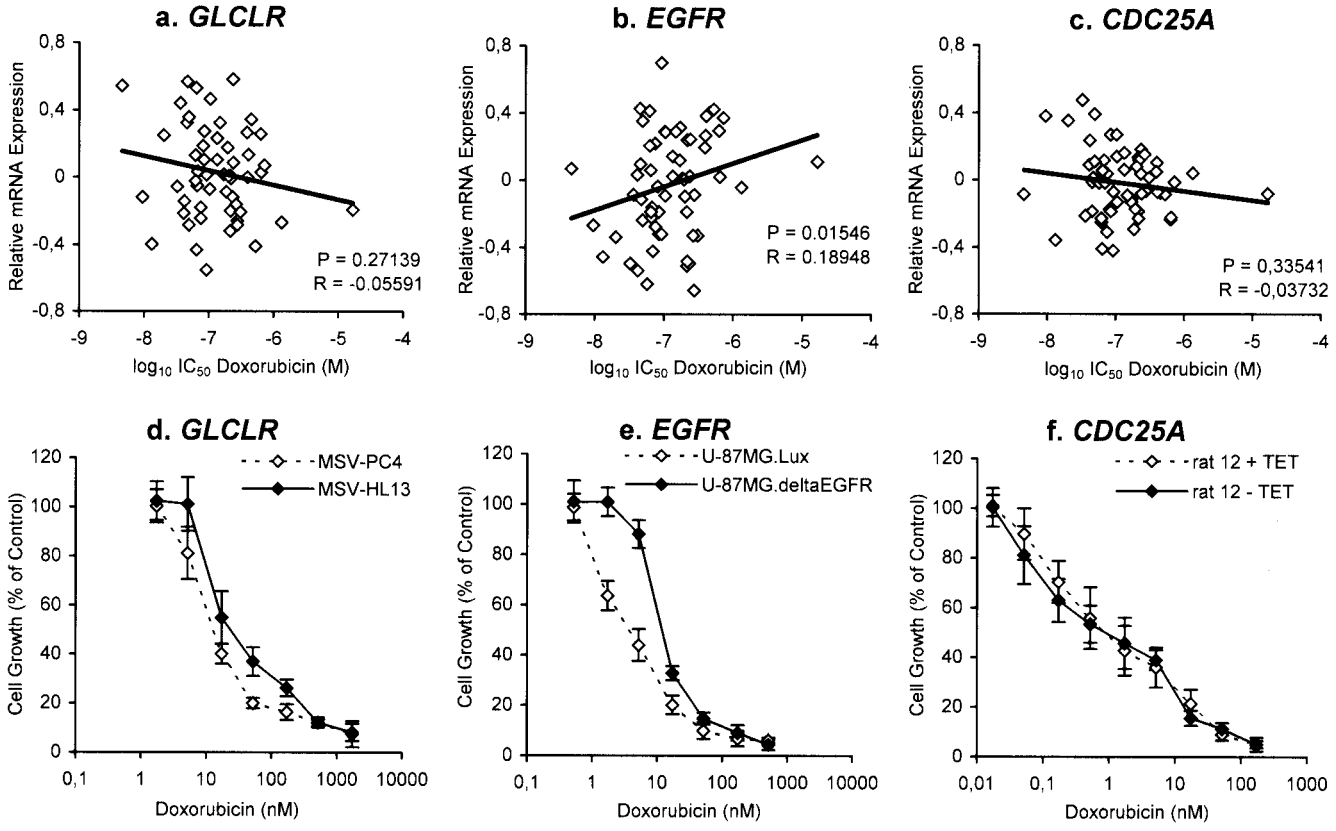

Fig. 4. Effect of doxorubicin on cell lines transduced with selected genes. For details see Fig. 3 legend.

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which mRNA expression correlated with the IC_{50} values of ART was lower than that for arteether or artemether. In the present investigation, the multidrug resistance-conferring genes *MDR1*, *MRP1*, and *BCRP* did not influence resistance to ART. Recently, we also found that *MDR1*-over-expressing doxorubicin-resistant CEM/ADR5000 cells or *MRP1*-over-expressing epirubicin-resistant CEM/E1 cells were not cross-resistant to ART (Efferth et al., 2002b). CEM/ADR5000 cells were used as controls in the present study. The lack of cross-resistance of *MRP1*-over-expressing cells was confirmed here using doxorubicin-resistant HL-60 cells. Now, we have extended this concept using *BCRP*-over-expressing cells. These findings give reason to hope that ART may be suited to treat refractory, multidrug-resistant tumors in a clinical setting. This is also consistent with ART's activity against multidrug-resistant *Plasmodium* strains (Price, 2000) and against ganciclovir-resistant human cytomegaloviruses (Efferth et al., 2002c). The correlations of the IC_{50} values for ART with the expression of DNA topoisomerase I should be interpreted with some caution. ART did not correlate with DNA topoisomerase I-inhibiting drugs (camptothecin, topothecan) (Efferth et al., 2002a). Since DNA topoisomerases are involved not only in multidrug resistance but also in proliferation, these correlations may reflect ART's relationship with proliferative index.

Some DNA damage and repair genes were correlated with ART activity, e.g., excision repair genes (*ERCC5*), damage recognition proteins of the high mobility group (*HMG*), and others. DNA repair represents a well known mechanism of drug resistance (Martin, 2001). DNA recognition proteins prevent repair of drug-induced DNA cross-links (Ohndorf et al., 1999). Previous investigations on ART's antimalarial activity focused only on protein alkylation and lipid peroxidation (Asawamahasakda et al., 1994; Berman and Adams, 1997). ART has not yet been shown to induce DNA damage. Therefore, the importance of the correlations of IC_{50} values for ART to DNA damage and repair gene expression remains unproven.

As shown previously, ART induces apoptosis in KG-1a leukemia cells (Efferth et al., 1996). In the present study, a number of apoptosis-regulating genes correlated with cellular response toward ART. This fits well with the concept that key molecules of the apoptosis cascade are important for chemotherapy-induced cell death (Johnstone et al., 2002).

A role for oncogenes and tumor suppressor genes for the cellular response to established anticancer drugs has been described (el Deiry, 1997). In the present investigation, several oncogenes and tumor suppressor genes were associated with sensitivity of tumor cells to ART. Interestingly, the p53 mutational status of the 55 NCI cell lines did not correlate with the IC_{50} values for ART. The loss of p53 function may, therefore, not result in ART resistance as previously shown

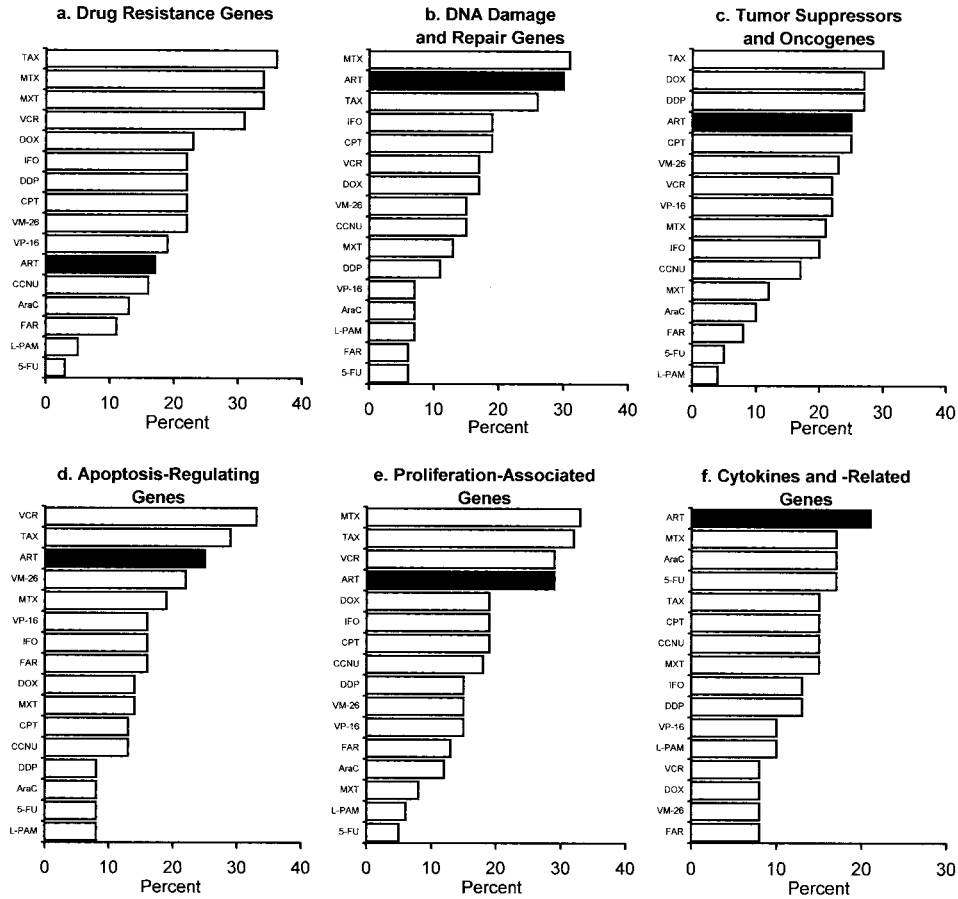

Fig. 5. Comparison of ART to established anticancer drugs. Shown are the percentages of genes of different functional categories in which mRNA expression significantly correlated to the IC_{50} values of the drugs in the NCI cell line panel. Abbreviations: AraC, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside); CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); CPT, camptothecin; DDP, *cis*-diamminedichloroplatinum(II) (cisplatin); DOX, doxorubicin; FAR, fludarabine; 5-FU, 5-fluorouracil; IFO, ifosfamide; L-PAM, melphalan; MTX, methotrexate; MXT, mitoxantrone; TAX, paclitaxel (Taxol); VCR, vincristine; VM-26, teniposide; VP-16, etoposide.

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for other anticancer drugs (O'Connor et al., 1997). Isogenic p53−/− cells derived from parental HCT116 cells and knockout cells of the p53-downstream gene $p21^{WAF1/CIP1}$ were not resistant to ART. After exposure to ART, p53 protein expression was induced in p53+/+ cells, indicating that ART does stimulate a p53-dependent stress-response pathway. Immunoblot and kinase assays of p53-dependent proteins that govern the progression through G_1 and G_2 cell cycle phases did not provide an explanation for a different regulation of these proteins in knock-in and knockout cells. Thus, p53-independent pathways must also be operative as a response pattern to ART. Interestingly, the expression of the *CDC25A* gene product that is a p53-independent regulator of G_1/S progression was down-regulated in knock-in and knockout cells after ART exposure. This is a clue that ART may act via *CDC25A*-dependent pathways in cancer cells.

EGFR expression correlates with resistance to established drugs (Wosikowski et al., 1997), and we also found a correlation with ART resistance. In addition, we used tumor cells transduced with an *EGFR* gene truncated in its extracellular domain

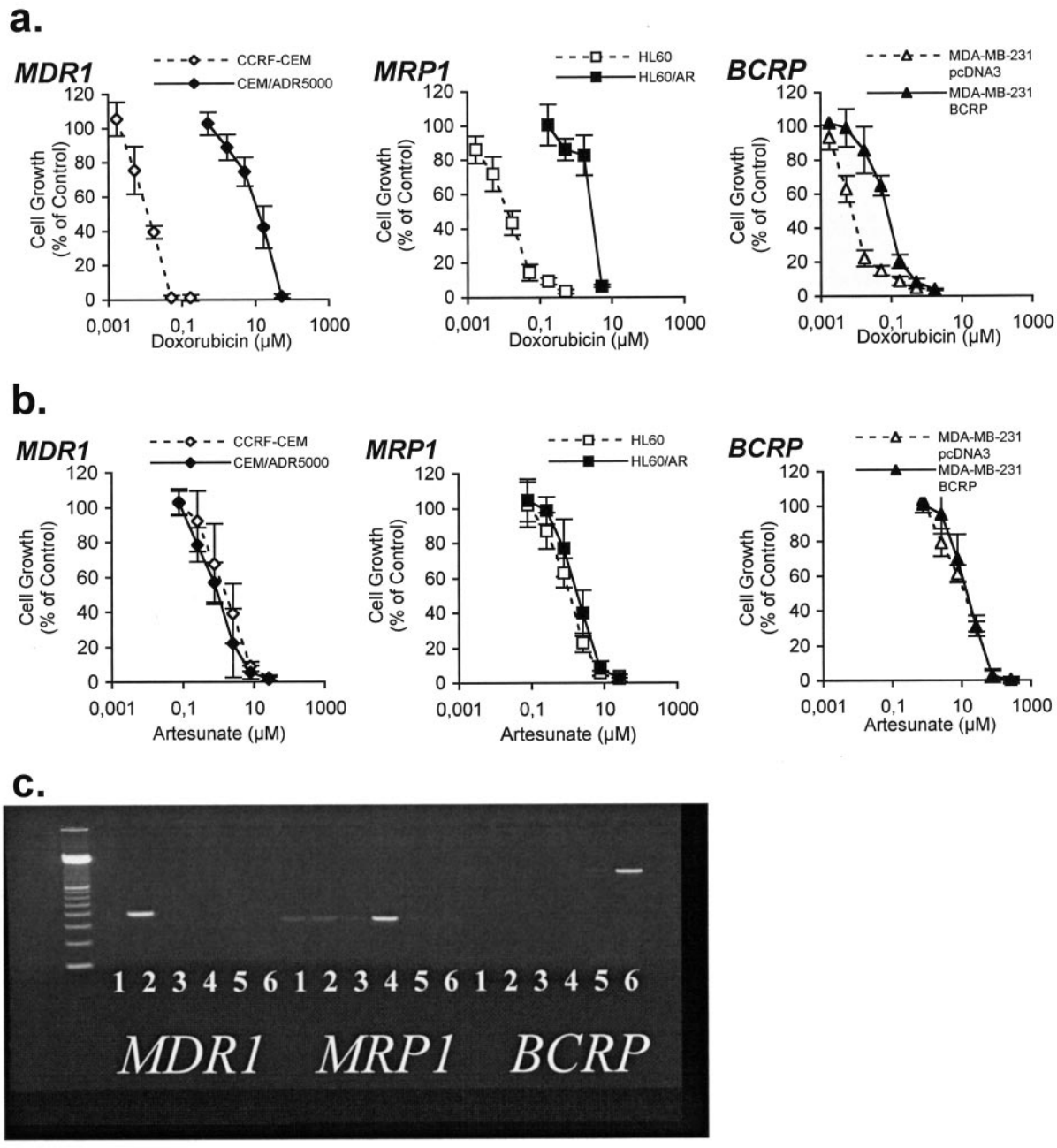

Fig. 6. Effect of ART on multidrug-resistant tumor cells. a, growth inhibition assays of *MDR1*-expressing CEM/ADR5000 cells, *MRP1*-expressing HL-60/AR cells, *BCRP*-expressing MDA-MB-231-BCRP clone 23 cells, and their drug-sensitive counterparts treated with doxorubicin. Control (100%) for each cell type represents cell growth without drug addition. Mean ± S.D. of two independent experiments with eight determinations. b, growth inhibition assays of sensitive and multidrug-resistant tumor cells treated with ART. For details see a. c, mRNA expression of the ABC transporter genes *MDR1* (*ABCB1*), *MRP1* (*ABCC1*), and *BCRP* (*ABCG2*) as measured by RT-PCR. Lane 1, sensitive CCRF-CEM; lane 2, doxorubicin-resistant CEM/ADR5000; lane 3, sensitive HL-60; lane 4, doxorubicin-resistant HL-60/AR; lane 5, sensitive MDA-MB-231-pcDNA3; lane 6, doxorubicin-resistant MDA-MD-231-BCRP clone 23.

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through a deletion of exons 2 to 7 (U-87MG.ΔEGFR). This truncated *EGFR* renders cells resistant to cisplatin, paclitaxel, and vincristine (Nagane et al., 1998). Transduction of the same tumor cell line with wild-type or tyrosine kinase-deficient *EGFR* genes did not result in drug resistance. In the present investigation, U-87MG.ΔEGFR cells were 13.6-fold more resistant to ART and 3.2-fold more resistant to doxorubicin than were U-87MG.Lux cells transduced with a mock control expression vector. The fact that EGFR confers resistance to ART and several other standard anticancer drugs confirms a general role for EGFR in drug resistance. This gene is, therefore, not a unique target for ART. Although *EGFR* and *CDC25A,* both of

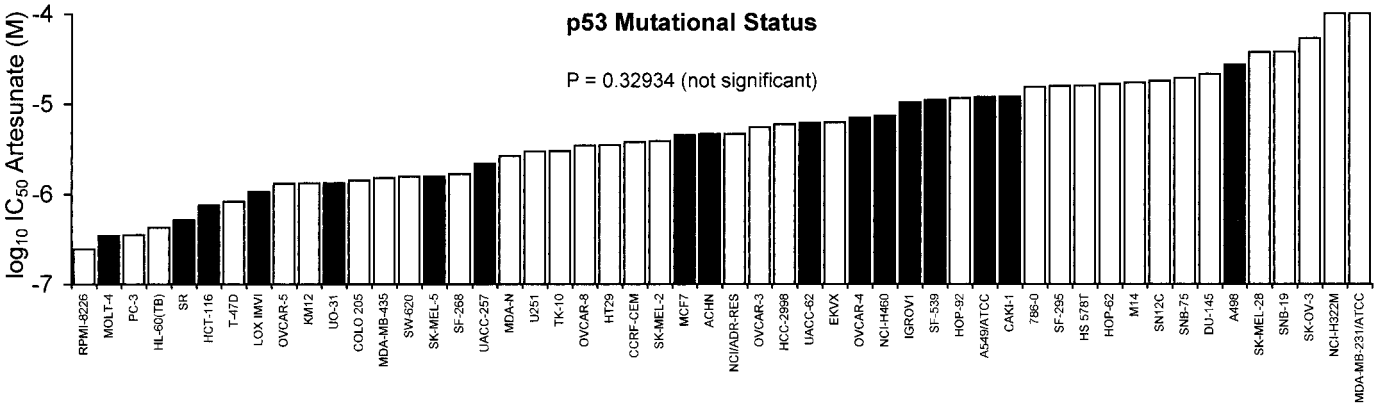

Fig. 7. Ranked order of $\log_{10}$ IC_{50} values for ART of 55 NCI cell lines in comparison to the mutational status of the tumor suppressor gene *p53* (solid bars, wild-type *p53*; open bars, mutated *p53*). The significance value has been calculated using the Mann-Whitney *U* test.

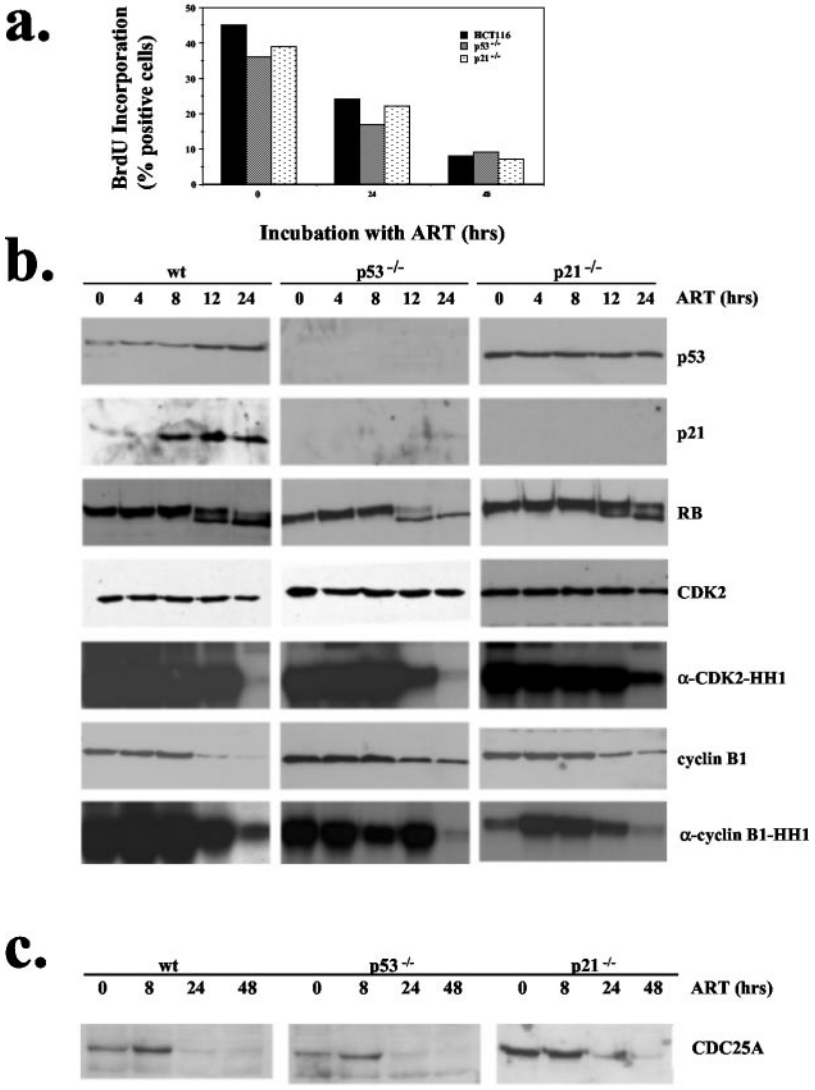

Fig. 8. Effect of ART on isogenic p53+/+ $p21^{WAF1/CIP1}$+/+, p53−/− $p21^{WAF1/CIP1}$+/+, and p53+/+ $p21^{WAF1/CIP1}$−/− HCT-116 human colon carcinoma cells. a, BrdU labeling after exposure with 10^{-5} M ART for 24 and 48 h. b, immunoblot and kinase assays of cell cycle-related proteins after exposure with 10^{-5} M ART for 4 to 24 h. c, immunoblot assays of p53-independent *CDC25A* gene product after exposure with 10^{-5} M ART for 8 to 48 h.

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which have been tested in our study, are considered as growth-promoting genes, they showed opposing effects. *EGFR* induced resistance, whereas *CDC25A* caused sensitivity to ART. This contradiction may be explainable by their effect on apoptosis. Besides its influence on proliferation, *EGFR* induces expression of the antiapoptotic *BCL-2* and *BCL-X_L* genes, down-regulates proapoptotic *BAX* expression, and inhibits apoptosis induced by diverse stimuli including cytostatic drugs (Nagane et al., 1998; Wang et al., 1999). *CDC25A* is a target gene of the proto-oncogene *MYC,* and *CDC25A* accelerates apoptosis (Galaktionov et al., 1996). Interestingly, *MYC* was also among the genes the expression of which correlated to sensitivity toward ART in our analysis.

Many of these 54 genes regulate proliferation, e.g., cell cycle genes (e.g., *CDK*s, *CDC*s), mitotic spindle motors (i.e., kinesins, dynein), and growth factors (e.g., *CTGF*, *VEGFC*). We have validated the role of *CDC25A* in a tetracycline repressor cell model. *CDC25A* is a rate-limiting controller for the transition from G_1 to S phase of the cell cycle. *CDC25A* dephosphorylates and activates the cyclin-*CDK* complexes that are active during G_1 (Blomberg and Hoffmann, 1999). Since the IC_{50} values of ART correlated with the G_0/G_1- and S-phase fractions in the 55 NCI cell lines, *CDC25A* may be an important regulator of cellular response to ART. This is compatible with the fact that ART is more cytotoxic in cell lines with a higher proliferative index. The fact that expression of *CDC25A* sensitized cells to ART but not to doxorubicin provides another hint that *CDC25A* might be a specific target for ART. This coincides with the down-regulation of *CDC25A* after ART exposure in p53 and p21 knock-in and knockout HCT-116 cell lines. Repression of *CDC25A* in the R12 Tet-off cell model reduces the entry from G_1 into S phase (Blomberg and Hoffmann, 1999). Although the precise mechanism is yet unknown, it could be that cells in the transition from G_1 to S phase are more vulnerable than cells in other phases of the cycle. Furthermore, it could be speculated that mechanisms in addition to S-phase entry may also be important. In a recent study, Wang et al. (2002) demonstrated that *CDC25A* interacts with *EGFR*. The authors linked *CDC25A* to *EGFR* downstream mitogenic signaling routes. Becuase both *CDC25A* and *EGFR* mRNA expression in the NCI cell line panel correlated significantly with the IC_{50} values for ART, this novel *CDC25A-EGFR* pathway may be important for cellular response to ART.

Cytokines and cytokine-associated genes were also tested in our approach. In addition to signaling proliferation and differentiation, cytokines contribute to the persistence of tumors following chemotherapeutic challenge (Löwenberg et al., 1993). Cytokines may influence drug resistance by stimulation of proliferation, apoptosis-regulating (i.e., *BCL-X_L*), drug resistance, and detoxification (e.g., *GST-π*) gene expression (Mizutani et al., 1995). All of these cytokine functions may also contribute to growth-inhibitory actions of ART on cancer cells.

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References

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, and Boyd MR (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* **48:**589–601.

Asawamahasakda W, Ittarat I, Pu YM, Ziffer H, and Meshnick SR (1994) Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrob Agents Chemother* **38:**1854–1858.

Batty KT, Davis TM, Thu LT, Binh TQ, Anh TK, and Ilett KF (1996) Selective high-performance liquid chromatographic determination of artesunate and alpha- and beta-dihydroartemisinin in patients with falciparum malaria. *J Chromatogr B Biomed Appl* **677:**345–350.

Berman PA and Adams PA (1997) Artemisinin enhances heme-catalysed oxidation of lipid membranes. *Free Radic Biol Med* **22:**1283–1288.

Blomberg I and Hoffmann I (1999) Ectopic expression of Cdc25A accelerates the G1/S transition and leads to premature activation of cyclin E- and cyclin A-dependent kinases. *Mol Cell Biol* **19:**6183–6194.

Brügger D, Herbart H, Gekeler V, Seitz G, Liu C, Klingebiel T, Orlikowsky T, Einsele H, Denzlinger C, Bader P, et al. (1999) Functional analysis of P-glycoprotein and multidrug resistance-associated protein-related multidrug resistance in AML-blasts. *Leuk Res* **23:**467–475.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, and Vogelstein B (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science (Wash DC)* **282:**1497–1501.

Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, and Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* **95:**15665–15670.

Efferth T, Davey M, Olbrich A, Rücker G, Gebhart E, and Davey R (2002b) Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. *Blood Cells Mol Dis* **28:**160–168.

Efferth T, Dunstan H, Sauerbrey A, Miyachi H, and Chitambar CR (2001) The anti-malarial artesunate is also active against cancer. *Int J Oncol* **18:**767–773.

Efferth T, Fabry U, and Osieka R (1997) Apoptosis and resistance to daunorubicin in human leukemic cells. *Leukemia* **11:**1180–1186.

Efferth T, Marschall M, Wang X, Huong SM, Hauber I, Olbrich A, Kronschnabl M, Stamminger T, and Huang ES (2002c) Activity of artesunate towards wild-type, recombinant GFP-expressing sensitive and ganciclovir-resistant human cytomegaloviruses. *J Mol Med* **80:**233–242.

Efferth T, Olbrich A, and Bauer R (2002a) mRNA expression profiles indicating the response of tumor cells to artesunate, arteether and artemether. *Biochem Pharmacol* **64:**617–623.

Efferth T, Rücker G, Falkenberg M, Manns D, Olbrich A, Fabry U, and Osieka R (1996) Apoptosis in KG-1a leukemic cells treated with investigational drugs. *Arzneim-Forsch* **46:**196–200.

el Deiry WS (1997) Role of oncogenes in resistance and killing by cancer therapeutic agents. *Curr Opin Oncol* **9:**79–87.

Funk JO, Waga S, Harry JB, Espling E, Stillman B, and Galloway DA (1997) Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev* **11:**2090–2100.

Galaktionov K, Chen X, and Beach D (1996) Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature (Lond)* **382:**511–517.

Hermeking H, Funk JO, Reichert M, Ellwart JW, and Eick D (1995) Abrogation of p53-induced cell cycle arrest by c-Myc: evidence for an inhibitor of p21$^{WAF1/CIP1/SDI1}$. *Oncogene* **11:**1409–1415.

Hien TT, Phu NH, Mai NT, Chau TT, Trang TT, Loc PP, Cuong BM, Dung NT, Vinh H, Waller DJ, et al. (1992) An open randomized comparison of intravenous and intramuscular artesunate in severe falciparum malaria. *Trans R Soc Trop Med Hyg* **86:**584–585.

Hochberg Y and Benjamini Y (1990) More powerful procedures for multiple significance testing. *Stat Med* **9:**811–818.

Huang HJS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, Huang CM, Gill GN, Wiley HS, and Cavenee WK (1997) The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* **272:**2927–2935.

Johnstone RW, Ruefli AA, and Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. *Cell* **108:**153–164.

Keselman HJ, Cribbie R, and Holland B (2002) Controlling the rate of Type I error over a large set of statistical tests. *Br J Math Stat Psychol* **55:**27–39.

Kimmig A, Gekeler V, Neumann M, Frese G, Handgretinger R, Kardos G, Diddens H, and Niethammer D (1990) Susceptibility of multidrug-resistant human leukemia cell lines to human interleukin 2-activated killer cells. *Cancer Res* **50:**6793–6799.

Löwenberg B, van Putten WLJ, Touw IP, Delwel R, and Santini V (1993) Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. *N Engl J Med* **328:**614–619.

Martin NM (2001) DNA repair inhibition and cancer therapy. *J Photochem Photobiol B Biol* **63:**162–170.

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Mizutani Y, Bonavida B, Koishihara Y, Akamatsu K, Ohsugi Y, and Yoshida O (1995) Sensitization of human renal cell carcinoma cells to cis-diamminedichloroplatinum(II) by anti-interleukin 6 monoclonal antibody or anti-interleukin 6 receptor monoclonal antibody. *Cancer Res* **55:**590–596.

Nagane M, Coufal F, Lin H, Bögler O, Cavenee WK, and Huang HJS (1996) A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res* **56:**5079–5086.

Nagane M, Levitzki A, Gazit A, Cavenee WK, and Huang HJ (1998) Drug resistance of human glioblastoma cells conferred by tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3 like proteases. *Proc Natl Acad Sci USA* **95:**5724–5729.

O'Brien M, Kruh GD, and Tew KD (2000) The influence of coordinate overexpression of glutathione phase II detoxification gene products on drug resistance. *J Pharmacol Exp Ther* **294:**480–487.

O'Connor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, Scudiero DA, Monks A, Sausville EA, Weinstein JN, et al. (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Res* **57:**4285–4300.

Ohndorf UM, Rould MA, He Q, Pabo CO, and Lippard SJ (1999) Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. *Nature (Lond)* **399:**708–712.

Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, and Boyd MR (1989) Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. *J Natl Cancer Inst* **81:**1088–1092.

Price RN (2000) Artemisinin drugs: novel antimalarial agents. *Expert Opin Investig Drugs* **9:**1815–1827.

Reiner A, Yekutieli D, and Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19:**368–375.

Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A, and Boyd MR (1990) Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst* **82:**1113–1118.

Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, et al. (2000) A gene expression database for the molecular pharmacology of cancer. *Nat Genet* **24:**236–244.

Volm M, Koomägi R, Mattern J, and Efferth T (2002) Protein expression profiles indicative for drug resistance of non-small cell lung cancer. *Br J Cancer* **87:**251–257.

Waldman T, Kinzler KW, and Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* **55:**5187–5190.

Walker DJ, Pitsch JL, Peng MM, Robinson B, Peters W, Bhisutthibhan J, and Meshnick SR (2000) Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii*. *Antimicrob Agents Chemother* **44:**344–347.

Wang Z, Wang M, Lazo JS, and Carr BI (2002) Identification of epidermal growth factor receptor as a target of Cdc25A protein phosphatase. *J Biol Chem* **277:**19470–19475.

Wang ZH, Ding MX, Yuan JP, Jim ML, Hao CF, Chew-Cheng SB, Ng HK, and Chew EC (1999) Expression of bcl-2 and Bax in *EGFR*-antisense transfected and untransfected glioblastoma cells. *Anticancer Res* **19:**4167–4170.

Weber HO, Samuel T, Rauch P, and Funk JO (2002) Human $p14^{ARF}$-mediated cell cycle arrest strictly depends on intact p53 signaling pathways. *Oncogene* **21:**3207–3212.

Wosikowski K, Schuurhuis D, Kops GJ, Saceda M, and Bates SE (1997) Altered gene expression in drug-resistant human breast cancer cells. *Clin Cancer Res* **3:**2405–2414.

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