Prediction of Broad Spectrum Resistance of Tumors towards Anticancer Drugs

Thomas Efferth,[1] V. Badireenath Konkimalla,[1] Yi-Fen Wang,[3] Axel Sauerbrey,[4] Silke Meinhardt,[5] Felix Zintl,[5] Jürgen Mattern,[2] and Manfred Volm[1]

Abstract

Purpose: Drug resistance is a major obstacle in cancer chemotherapy. Although the statistical probability of therapeutic success is known for larger patient groups from clinical therapy trials, it is difficult to predict the individual response of tumors. The concept of individualized therapy aims to determine *in vitro* the drug response of tumors beforehand to choose effective treatment options for each individual patient.

Experimental Design: We analyzed the cross-resistance profiles of different tumor types (cancers of lung, breast, and colon, and leukemia) towards drugs from different classes (anthracyclines, antibiotics, *Vinca* alkaloids, epipodophyllotoxins, antimetabolites, and alkylating agents) by nucleotide incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Hierarchical cluster analysis and COMPARE analyses were applied.

Results: Tumors exert broad resistance profiles, e.g., tumors resistant to one drug tend to also be resistant to other drugs, whereas sensitive tumors reveal sensitivity towards many drugs. Interestingly, the broad spectrum resistance phenotype could reliably be predicted by doxorubicin alone. Expression of the ATP-binding cassette transporter P-glycoprotein (*ABCB1, MDR1*) and the proliferative activity of tumors were identified as underlying mechanisms of broad spectrum resistance. To find novel compounds with activity against drug-resistant tumors, a database with 2,420 natural products was screened for compounds acting independent of P-glycoprotein and the proliferative state of tumor cells.

Conclusions: Tumors exert cross-resistance profiles much broader than the classical multidrug resistance phenotype. Broad spectrum resistance can be predicted by doxorubicin due to the multifactorial mode of action of this drug. Novel cytotoxic compounds from natural resources might be valuable tools for strategies to bypass broad spectrum resistance.

Tracing the rise of tumor chemotherapy from the discovery of the first anticancer drug, nitrogen mustard (1), to the advent of molecular approaches sheds light on the problem of drug resistance, which has dogged oncology for the past six decades. Due to the modest tumor specificity of many anticancer drugs, normal tissues are also damaged leading to severe side effects. This prevents the application of sufficiently high doses to eradicate less sensitive tumor cell populations. Thereby, tumors develop drug resistance that lead to treatment failure and fatal consequences for patients. Novel strategies to broaden the narrow therapeutic range by separating the effective dose and the toxic dose would be of great benefit for the improvement of cancer chemotherapy.

A number of concepts have been developed to help in the war against cancer, e.g., modulators of drug resistance, non–cross-resistant drug derivatives, high-dose chemotherapy, hematopoietic stem cell transplantation, and supportive gene therapy protocols. Another option is the a priori diagnosis of drug-resistant tumors. The question arises as to which particular cytotoxic agent and which combination of substances are the most suited for an individual tumor. Although the statistical probability of therapeutic success is well known for larger groups of patients from clinical therapy trials, it is, however, not possible to predict how an individual tumor will respond to chemotherapy. Although clinicopathologic prognostic factors such as tumor size, lymph node, and far distance metastases are valuable for the determination of prognosis of larger cohorts, they are less helpful for the development of personalized therapy options. Biomarkers are desired to stratify patients into groups of different likelihood of the tumors' responsiveness or of toxicity in normal organs. The concept of individualized therapy itself traces back to the 1950s (2), and sensitivity testing of tumors was thriving in the 1970s and

Authors' Affiliations: [1]German Cancer Research Center, [2]OncoSurgery, University of Heidelberg, Heidelberg, Germany, [3]Institute of Botany, Key State Laboratories, Chinese Academy of Sciences, Kunming, Yunnan, China, [4]Helios Clinics, Children's Hospital, Erfurt, Germany, and [5]Department of Pediatrics, University of Jena, Jena, Germany

Received 10/3/07; revised 1/8/08; accepted 1/8/08.

Grant support: The Dietmar Hopp Stiftung, Germany (V.B. Konkimalla); the German Cancer Research Center, Heidelberg, Germany; the University of Jena, Germany; and the Institute of Botany, Chinese Academy of Science, Kunming, China.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Thomas Efferth, Pharmaceutical Biology (C015), German Cancer Research Center, Im Neuenheimer Feld 280, 69120, Heidelberg, Germany. Phone: 49-6221-423426; Fax: 49-6221-423433; E-mail: t.efferth@dkfz.de.

doi:10.1158/1078-0432.CCR-07-4525

Table 1. Correlation between response to 10 different antitumor agents in 59 biopsies of different tumor types

		Daunorubicin	Dactinomycin	Bleomycin	5-Fluorouracil
Doxorubicin	*R* value	0.711	0.657	0.338	0.419
	P value	5.1×10^{-14}	1×10^{-13}	8.9×10^{-5}	1.4×10^{-6}
Daunorubicin	*R* value		0.665	0.331	0.453
	P value		1.8×10^{-12}	3.1×10^{-4}	1.1×10^{-6}
Dactinomycin	*R* value			0.388	0.455
	P value			8.6×10^{-6}	1.8×10^{-7}
Bleomycin	*R* value				0.291
	P value				6.3×10^{-4}
5-Fluorouracil	*R* value				
	P value				
Cytarabin	*R* value				
	P value				
Methotrexate	*R* value				
	P value				
Procarbazin	*R* value				
	P value				
Mitzopodozide	*R* value				
	P value				
Triaziquone	*R* value				
	P value				

NOTE: Drug sensitivity was measured using the nucleotide incorporation assay, and Kendall's τ test was used for correlation analysis.
*Incorporation of tritium-labeled thymidine in untreated control cells.

1980s (3–7). The idea was to determine the *in vitro* response of tumors to cytotoxic agents beforehand, in order to choose the most effective treatment options for each individual patient clinically.

Unfortunately, at that time, it was not possible to translate the results from the bench to the bedside. Among the technical difficulties hampering routine application of sensitivity tests in the clinic, one surprising observation was that tumors resistant to one drug also exerted resistance to other chemically and functionally unrelated drugs. In an era in which most oncologists were convinced of the benefit of a drug combination regimen to overcome resistance to single drugs, this finding experienced criticism.

The aim of the present investigation was to analyze the cross-resistance profile of clinical tumor samples *in vitro*. For this reason, we investigated different tumor types and two different methods to determine drug sensitivity and resistance. Cytotoxic drugs from different classes were used (anthracyclines, antibiotics, *Vinca* alkaloids, epipodophyllotoxins, antimetabolites, and alkylating agents). Hierarchical cluster analysis was applied to evaluate whether resistance to multiple drugs could be predicted by one single drug alone, doxorubicin.

As a second step, the cross-resistance profiles were analyzed in sensitive and resistant cell lines from different tumor types to study the underlying mechanisms. In an effort to find new treatment possibilities, novel cytotoxic compounds with activity against otherwise drug-resistant tumor cells were investigated.

Materials and Methods

Cell lines

Human CCRF-CEM leukemia cells were maintained in RPMI medium (Life Technologies) supplemented with 10% FCS in a humidified 7% CO_2 atmosphere at 37°C. Cells were passaged twice weekly. All experiments were done with cells in the logarithmic growth phase. The multidrug resistance gene 1 (*ABCB1*, *MDR1*)–expressing CEM/ADR5000 (8) were maintained in 5,000 ng/mL of doxorubicin. CEM/ADR5000 cells have previously been shown to selectively express *MDR1* (*ABCB1*), but none of the other ATP-binding cassette (ABC) transporters (9).

Patients and tumors

Fifty-nine tumors of diverse tumor localization were obtained from the Department of General Surgery, the Department of Gynecology and Obstetrics, and the Department of Urology (University of Heidelberg, Germany; Supplementary Table S1). Thirty-eight lung tumors were obtained from the Chest Hospital, Heidelberg Rohrbach (Supplementary Table S2). Twenty-one leukemia samples were obtained from the Department for Pediatrics, University of Jena, Jena, Germany (Supplementary Table S3). All tumors were not pretreated with chemotherapy at the time point of *in vitro* testing. Informed consent was obtained from each patient.

Detection of tumor resistance *in vitro*

Drugs. Doxorubicin, daunorubicin, doxorubicinol, epirubicin, epirubicinol, idarubicin, and idarubicinol were provided by Pharmacia and Upjohn. Vincristine, vinblastine, vindesine, vinorelbine, etoposide, etoposide phosphate, paclitaxel, and methotrexate were obtained from Bristol-Myers. Cytarabin and bleomycin were a gift from Mack (Illertissen, Germany). The other standard drugs were from MSD (dactinomycin), Lederle (5-fluorouracil), and Sanofi-Aventis (docetaxel). L-Alanosine was a gift from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (NCI), procarbazin from Hoffmann la Roche, mitopodozide from Novartis, and triaziquone from Bayer. The traditional Chinese medicine (TCM)–derived drugs cephalotaxine, homoharringtonine, berberine, and cantharidin were purchased from Sigma-Aldrich and artesunate from Saokim. The other natural products were isolated from their medicinal plants of origin as described (10, 11).

Nucleotide incorporation assay. For determining the drug resistance of tumors, we used a short-term *in vitro* assay that has been described previously (5, 6). Its basic feature is the measurement of changes in the incorporation of radioactive nucleic acid precursors into tumor cells

Table 1. Correlation between response to 10 different antitumor agents in 59 biopsies of different tumor types (Cont'd)

Cytarabine	Methotrexate	Procarbazin	Mitopodozide	Triaziquone	TdR (cpm)
0.260	0.093	0.0420	0.372	0.409	-0.384
0.002	n.s.	n.s.	1.6×10^{-5}	2.9×10^{-6}	2.1×10^{-5}
0.308	0.066	0.062	0.378	0.443	-0.338
7.9×10^{-4}	n.s.	n.s.	3.9×10^{-5}	2.2×10^{-6}	4.0×10^{-4}
0.267	0.067	0.060	0.507	0.428	-0.343
0.002	n.s.	n.s.	7.0×10^{-9}	1.0×10^{-6}	1.3×10^{-4}
0.274	0.284	0.285	0.355	0.204	-0.033
0.001	0.001	7.8×10^{-4}	4.2×10^{-5}	0.012	n.s.
0.286	0.254	0.036	0.201	0.223	-0.248
8.3×10^{-4}	0.003	n.s.	0.012	0.007	0.004
	0.332	0.262	0.225	0.352	-0.137
	1.7×10^{-4}	0.002	0.007	6.3×10^{-5}	n.s.
		0.466	0.130	0.191	0.138
		2.0×10^{-7}	n.s.	0.020	n.s.
			0.270	0.207	0.102
			0.001	0.011	n.s.
				0.303	-0.093
				3.9×10^{-4}	n.s.
					-0.192

after the addition of cytotoxic drugs. We found that anthracyclines (i.e., doxorubicin) could be used as reference compounds for broad spectrum resistance. Tumors were defined as being sensitive or resistant depending on whether nucleotide uptake was inhibited by more than or less than 65% at a concentration of 10 μg/mL doxorubicin. This threshold was based on prior clinical correlations (5).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Peripheral blood mononuclear cells were separated by density gradient centrifugation (Lymphoprep, Nycomed Pharma). Cytotoxicity was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (12). Briefly, 96-well microculture plates contained a total volume of 80 μL cell suspension (1×10^6/mL) in complete medium. Cells were either pretreated or drugs were directly added to the wells in a final volume of 20 μL. All drug concentrations and untreated controls were set up 3-fold. After incubation in humidified air containing 5% CO_2 for 4 days at 37°C, 10 μL of MTT solution (Sigma) was added for 6 h. MTT is reduced to a colored formazan by living cells but not by dead cells. The formazan crystals were dissolved with 100 μL of 0.04 N HCl-isopropanol. Absorbance of the wells was determined with a microplate reader (Spectra, SLT) at 570 nm test wavelength and 690 nm reference wavelength. The drug concentration required to kill 50% of the cells as compared with control cell survival (IC_{50}) was calculated from the dose-response curve.

Sulforhodamine B assay. The determination of drug sensitivity in the NCI cell lines by the sulforhodamine B assay has been previously reported (13). The 50% inhibition concentration (IC_{50}) values for standard anticancer drugs have been deposited in the NCI database.[6] The IC_{50} values of 531 natural products are also deposited in the NCI database.

Statistical analysis

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

The Fisher exact test was used as an implement of the WinSTAT program (Kalmia) to prove bivariate frequency distributions for pairs of nominal scaled variables for dependencies.

Objects were classified by the 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, in which 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. Cluster analyses applying complete linkage methods were done by means of the WinSTAT program (Kalmia). Missing values are 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 the distances of all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean = 0 and a variance = 1. Tumors or cell lines

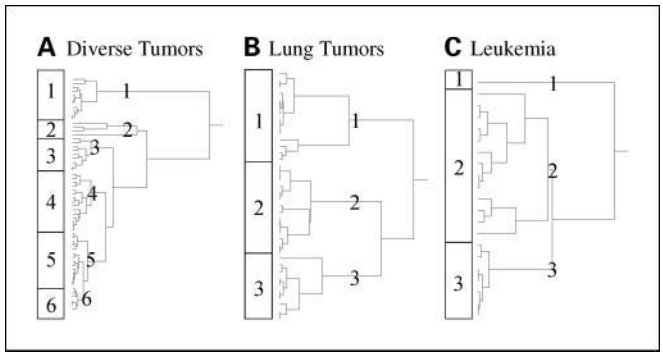

Fig. 1. Hierarchical cluster analysis (complete linkage method) of sensitivity testing results of clinical tumor specimens towards different antitumor drugs. Dendrograms obtained from clustering of (*A*) 59 diverse tumors, (*B*) 38 lung cancers, and (*C*) 21 leukemia samples. Bottom, separation of clusters and comparison to chemosensitivity towards doxorubicin. The sensitivity of tumor specimens to antitumor drugs was determined by the nucleotide incorporation assay (*A* and *B*) and MTT assay (*C*), respectively.

[6] http://dtp.nci.nih.gov

Table 2. Separation of clusters of tumors obtained by the hierarchical cluster analysis shown in Fig. 1 in comparison with sensitivity towards doxorubicin (A and B) and daunorubicin (C)

	Sensitive	Resistant	Fisher exact test
(A) Diverse tumors			
Clusters 1, 5, and 6	3	28	
Clusters 2, 3, and 4	21	7	$P = 2.54 \times 10^{-7}$
(B) Lung tumors			
Clusters 1 and 3	20	4	
Cluster 2	4	10	$P = 1.18 \times 10^{-3}$
(C) Leukemia			
Clusters 1 and 2	0	14	
Cluster 3	6	1	$P = 1.29 \times 10^{-4}$

NOTE: Fixed values of 65% of control for the nucleotide incorporation assay (A, B) and 0.15 μg/mL (C) for the MTT assay were used as cutoffs to separate tumor cell lines as being "sensitive" or "resistant". Statistical calculation was done by means of Fisher exact test.

with the most identical features appear side by side in the dendrogram.

COMPARE analyses were done using a panel of 60 cell lines of different tumor types from the NCI. The methodology and the cell lines have been described in detail previously (14, 15). Briefly, every gene of the NCI microarray database was ranked for similarity of its mRNA expression to the IC_{50} values for doxorubicin. The sensitivity to doxorubicin was determined by the sulforhodamine B assay (13), whereas the mRNA expression was determined by microarray analyses (16, 17). The data were stored in the NCI database.[6] To derive COMPARE rankings, a scale index of Pearson's correlation coefficients (*R* values) was created. This approach has been used to identify possible candidate genes associated with the response of the 60 cell lines to doxorubicin. In the standard COMPARE approach, greater mRNA expression in cell lines correlates with enhanced drug sensitivity, whereas in reverse COMPARE analyses, greater mRNA expression in cell lines indicates drug resistance.

Results

The objective of this study was to evaluate the cross-resistance profiles of tumors. As a first step, we analyzed 59 tumors of different origins (Supplementary Table S1) for their resistance *in vitro* to cytotoxic drugs from the classes of anthracyclines (doxorubicin, daunorubicin), antibiotics (dactinomycin, bleomycin), antimetabolites (5-fluorouracil, methotrexate), epipodophyllotoxins (mitopodozide), and alkylating agents (procarbazine, triaziquone) by means of the nucleotide incorporation assay. As can be seen in Table 1, the response of the tumors to doxorubicin correlated significantly with the response to all other drugs tested (Kendall's τ test).

To gain more insight into the drug resistance profiles, we decided to perform hierarchical cluster analysis, which may be more suited for an integrated understanding of drug resistance. We subjected all drugs analyzed except for doxorubicin to hierarchical cluster analysis, in order to find the profiles indicative for drug resistance (Fig. 1A). We divided the dendrogram into six clusters and correlated them with the resistance data for doxorubicin, which were not included as variables into the cluster analysis. Sensitive and resistant tumors were separated in the clusters ($P = 2.5 \times 10^{-7}$; Table 2). Clusters 1, 5, and 6 ($n = 31$) were enriched with resistant tumors (54%), whereas clusters 2, 3, and 4 ($n = 28$) were enriched with sensitive ones.

To further explore this phenomenon in a group of tumors of the same tumor type, we investigated 38 lung cancers for their response *in vitro* to doxorubicin, 5-fluorouracil, and hydroxy-cyclophosphamide (the *in vitro* active form of cyclophosphamide; Supplementary Table S2). Again, we performed Kendall's τ rank correlation test and found a statistically significant relationship between resistance to doxorubicin and resistance to 5-fluorouracil and hydroxy-cyclophosphamide (Table 3). Hierarchical cluster analysis using the data for 5-fluorouracil and hydroxy-cyclophosphamide allowed the separation of three clusters (Fig. 1B). Although clusters 1 and 3 contained mainly 5-fluorouracil and hydroxy-cyclophosphamide–sensitive tumors, which were also sensitive to doxorubicin, cluster 2 was enriched with tumors resistant to these drugs ($P = 1.2 \times 10^{-3}$; Table 2).

To exclude the possibility that the cross-resistance profiles obtained were biased by the method used for the determination of drug resistance, we tested a third set of tumors using the MTT assay. Twenty-one clinical leukemia samples were used to determine their responsiveness *in vitro* to daunorubicin, etoposide, cytarabine, and 6-thioguanine (Supplementary Table S3). Because doxorubicin is clinically less important for the treatment of leukemia, we used the closely related daunorubicin instead. As shown in Table 4, the response of the leukemia samples to daunorubicin correlated significantly with the other drugs investigated (Kendall's τ test). If the data for etoposide, 6-thioguanine, and cytarabine were subjected to hierarchical cluster analysis, three major clusters would appear and show a significant relationship with daunorubicin ($P = 1.3 \times 10^{-4}$; Fig. 1C; Table 2).

Table 3. Correlation between responses to three different antitumor agents in 38 biopsies of lung tumors

		5-Fluorouracil	Cyclophosphamide	TdR*
Doxorubicin	*R* value	0.373	0.290	-0.421
	P value	4.8×10^{-4}	0.005	9.8×10^{-5}
5-Fluorouracil	*R* value		0.258	-0.496
	P value		0.011	5.9×10^{-6}
Hydroxy-cyclophosphamide	*R* value			-0.389
	P value			3.0×10^{-4}

NOTE: Drug sensitivity was measured using the nucleotide incorporation assay, and Kendall's τ test was used for correlation analysis.
*Incorporation of tritium-labeled thymidine in untreated control cells.

Table 4. Correlation between response to four different antitumor agents in 21 leukemia samples

		Vincristine	Etoposide	Cytarabine	6-Thioguanine
Daunorubicin	*R* value	0.494	0.613	0.350	0.378
	P value	0.003	7.9×10^{-5}	0.016	0.001
Vincristine	*R* value		0.308	0.441	0.596
	P value		0.048	0.009	6.4×10^{-4}
Etoposide	*R* value			0.102	0.096
	P value			n.s.	n.s.
Cytarabine	*R* value				0.290
	P value				0.0414

NOTE: Drug sensitivity was measured using the MTT assay, and Kendall's τ test was used for correlation analysis.
Abbreviation: n.s., not significant ($P > 0.05$).

Next, we did COMPARE analyses between the IC_{50} values of 60 tumor cell lines of the NCI for doxorubicin and the microarray-based genome-wide mRNA expression of these cell lines in order to identify gene-drug relationships. Interestingly, *MDR1* (*ABCB1*) was the only gene whose expression correlated with a COMPARE correlation coefficient >0.6 with the IC_{50} values for doxorubicin, indicating that *MDR1* (*ABCB1*) plays an important role for cellular response to this drug.

To prove this in more detail, we used the CEM/ADR5000 leukemia cell line overexpressing *MDR1*/P-glycoprotein and its

Table 5. Cross-resistance profile of multidrug-resistant human CEM/ADR5000 leukemia cells to established cytostatic drugs and natural products derived from TCM

Compound	IC_{50} value		Degree of resistance
	CCRF-CEM	CEM/ADR5000	
Anthracyclines			
Doxorubicin	11.8 (± 1.9) nmol/L	12.2 (± 54.2) μmol/L	1,036
Epirubicin	21.7 (± 3.1) nmol/L	10.5 (± 3.9) μmol/L	484
Idarubicin	5.7 (± 1.0) nmol/L	39.1 (± 9.8) nmol/L	6.9
Doxorubicinol	467 (± 103) nmol/L	178.5 (± 35.8) μmol/L	382
Epirubicinol	304 (± 35) nmol/L	47.9 (± 19.9) μmol/L	158
Idarubicinol	8.4 (± 2.4) nmol/L	13.7 (± 1.7) nmol/L	1.6
Vinca alkaloids			
Vincristine	1.7 (± 0.1) nmol/L	1,043 (± 145) nmol/L	613
Vinblastine	6.3 (± 0.2) nmol/L	212 (± 18) nmol/L	34
Vindesine	80 (± 3.3) nmol/L	1,124 (± 60) nmol/L	14
Vinorelbine	0.6 (± 0.1) nmol/L	106 (± 45) nmol/L	177
Taxanes			
Docetaxel	0.4 (± 0.1) nmol/L	175 (± 17) nmol/L	438
Paclitaxel	3.7 (± 0.4) nmol/L	741 (± 137) nmol/L	200
Epipodophyllotoxins			
Etoposide	85 (± 20) nmol/L	1,563 (± 139) nmol/L	18
Etoposide phosphate	69 (± 7) nmol/L	748 (± 90) nmol/L	11
Antimetabolites			
Methotrexate	14 (± 2) nmol/L	10 (± 2) nmol/L	0.71
L-Alanosine	4.3 (± 1.7) μmol/L	3.0 (± 0.6) μmol/L	0.7
Natural products derived from TCM			
Cephalotaxine*	15 (± 6.1) μmol/L	139.9 (± 37.2) μmol/L	9.3
Berberine*	26 (± 3.3) μmol/L	158 (± 9.7) μmol/L	6.1
Homoharringtonine*			
Maesopsin †	5.3 (± 2) μmol/L	12.3 (± 2.6) μmol/L	2.3
(*E*)-3-(4-Hydroxyphenyl)-[2-(4-hydroxyphenyl)-ethyl]-prop-2-enamide ‡	15.8 (± 3.8) μmol/L	24.3 (± 4.2) μmol/L	1.5
Maslinic acid ‡	7.1 (± 0.9) μmol/L	9.0 (± 1.1) μmol/L	1.3
Cantharidin*	19.6 (± 2.6) μmol/L	17.7 (± 3.1) μmol/L	0.9
3-(2-Hydroxyethyl)-1*H*-indole-5-*O*-β-D-glucopyranoside ‡	29.7 (± 3.6) μmol/L	27 (± 4.7) μmol/L	0.9
Kaemperol †	14.0 (± 17.0) μmol/L	10.2 (± 5) μmol/L	0.7
Artesunate*	1.8 (± 1.2) μmol/L	1.2 (± 0.7) μmol/L	0.7

NOTE: Dose response curves obtained by means of a cell growth inhibition assay were used to calculate IC_{50} values (mean ± SD).
*Data from ref. (29).
† Data from ref. (10).
‡ Data from ref. (11).

parental cell line, CCRF/CEM to investigate cross-resistance profiles. As shown in Table 5, CEM/ADR5000 cells were >1,000-fold resistant to the selecting agent, doxorubicin. They were also highly cross-resistant to the anthracycline epirubicin (484-fold) but less cross-resistant to idarubicin (6.9-fold). We also tested the anthracycline metabolites doxorubicinol, epirubicinol, and idarubicinol, which revealed cross-resistance to a lesser degree than the nonmetabolized parental drugs (range from 1.6- to 382-fold). Cross-resistance to the *Vinca* alkaloids vincristine, vinblastine, vindesine, and vinorelbine was in a range of 14-fold to 613-fold and to the taxanes paclitaxel and docetaxel in a range of 200-fold to 438-fold. The degrees of resistance to the epipodophyllotoxines etoposide and etoposide phosphate were lower (18-fold and 11-fold, respectively). Multidrug-resistant CEM/ADR5000 cells did not exhibit cross-resistance to the antimetabolites methotrexate and L-alanosine (Table 5), indicating that the *MDR1* (*ABCB1*) gene does not confer resistance to all drugs and that other factors are likely to act in concert with P-glycoprotein to generate the clinically observed phenotype of broad spectrum resistance.

Therefore, we analyzed the effect of cell proliferation as another independent factor of response to cytostatic drugs. A variable to measure the proliferative activity of clinical tumor samples was the incorporation rate of tritiated thymidine into DNA (radioactive cpm). The cpm values of the 59 clinical tumors of different origin as well as of the 38 lung cancers significantly correlated with response to doxorubicin, daunorubicin, dactinomycin, bleomycin, 5-fluorouracil, and cyclophosphamide at a significance level of $P = 0.05$ and $R > 0.2$ (Tables 1 and 3). The higher the cpm values were, the higher the inhibition rates by cytotoxic drugs, e.g., the more sensitive the tumors were.

In an effort to identify novel compounds with activity against otherwise drug-resistant tumor cells, we analyzed natural products derived from medicinal plants used in TCM in CCRF/CEM and CEM/ADR5000 cells. Interestingly the multidrug-resistant cells revealed an either low degree of cross-resistance (cephalotaxine, berberine, homoharringtonine, maesopsin), no clear cross-resistance [(*E*)-3-(4-hydroxyphenyl)-[2-(4-hydroxyphenyl)-ethyl]-prop-2-enamide, *N*-*p*-coumaryl tyramine, maslinic acid] or even enhanced sensitivity towards the natural products from TCM [cantharidin, tetracentronside, 3-(2-hydroxyethyl)-1H-indole-5-*O*-β-D-glucopyranoside, kaempferol, artesunate; Table 5].

Finally, we used a recently established database of 531 chemically characterized compounds from medicinal plants used in TCM (18) and correlated the IC_{50} values of the 60 NCI tumor cell lines for these 531 natural products with the accumulation data for rhodamine 123 as a functional assay for P-glycoprotein and with the cell doubling times as a variable of proliferation. Although the IC_{50} values correlated with rhodamine 123 accumulation for only 18 compounds (3%; Supplementary Table S4), 162 natural products were significantly associated with the cell doubling times of the cell lines (31%; Supplementary Table S5), indicating that natural products might be a rich source for novel drug candidates with activity against P-glycoprotein and proliferation-associated drug resistance.

Discussion

In the present investigation, we observed that tumors resistant to doxorubicin were also resistant to other cytotoxic drugs. This indicates a pleiotropic drug resistance phenotype. The fact that broad spectrum cross-resistance profiles were found in different tumor types speaks for the general relevance of this phenomenon. Broad spectrum resistance was observed by using two independent assays, e.g., the nucleotide incorporation assay and the MTT assay. This excludes the possibility that the observed cross-resistances were biased by artifacts related to assay-specific effects.

In the past years, the concept of multidrug resistance has been developed. Drug pumps from the ABC transporter type have been identified, i.e., P-glycoprotein (ABCB1/MDR1), multidrug-resistance–related proteins (ABCC1-6, MRP1-6), and breast cancer resistance protein (ABCG2/BCRP; refs. 19, 20). ABC transporters confer resistance to many antitumor agents. Indeed, our COMPARE analysis of microarray-based mRNA expression profiling point to P-glycoprotein/MDR1 as the most important factor for broad spectrum resistance. P-glycoprotein is known to extrude drugs of different classes including anthracyclines, antibiotics, *Vinca* alkaloids, epipodophyllotoxins, and taxanes, but not antimetabolites and alkylating agents. In our analysis, we found that resistance of tumors to doxorubicin correlated with resistance to 5-fluorouracil and hydroxy-cyclophosphamide, both of which were not transported by P-glycoprotein. This indicates that P-glycoprotein could only partly explain the broad spectrum resistance seen in our analyses and that other mechanisms may also contribute to this phenomenon.

Most established cytotoxic drugs suffer from a lack of tumor specificity. They act against proliferating cells. As the proliferative activity of tumors generally is higher than in normal organs, antitumor drugs preferentially kill proliferating tumor cells. Nevertheless, proliferating normal cells are also affected, i.e., bone marrow, gastrointestinal mucosa, hair cells, germ cells, etc. This leads to severe side effects of chemotherapy (myelosuppression, mucositis, alopecia, sterility, etc.). Therefore, we analyzed the relevance of proliferative activity of clinical tumors for the responsiveness towards cytotoxic drugs. The rate of incorporation of tritium-labeled thymidine in untreated control samples served as a variable for the proliferative activity. The fact that the sensitivity to most drugs tested (including 5-fluorouracil and hydroxy-cyclophosphamide) correlated with high proliferative activity indicates that the tumor's growth rate represents another important determinant of broad spectrum resistance independent of P-glycoprotein.

Our cluster analyses revealed that clusters of sensitive or resistant tumors could be predicted by one single drug, doxorubicin. This speaks for the high predictive power of doxorubicin to detect broad spectrum resistance. The pleiotropic modes of action of doxorubicin might explain why doxorubicin is capable of predicting broad spectrum resistance.

1. Doxorubicin is transported by P-glycoprotein and, therefore, is involved in the multidrug resistance phenotype. Therefore, doxorubicin can predict response to antibiotics, *Vinca* alkaloids, and epipodophyllotoxins.
2. Doxorubicin inhibits DNA topoisomerase II which is necessary for cell division. The activity of doxorubicin is dependent on the proliferative activity of tumor cells. Therefore, doxorubicin may predict the responsiveness of tumors to other drugs which also act in a proliferation-dependent manner such as antimetabolites, i.e., 5-fluorouracil.

3. The inhibition of DNA topoisomerase II by doxorubicin induces DNA strand breaks. Because alkylating agents also damage DNA, doxorubicin may predict the response to alkylators such as hydroxy-cyclophosphamide.
4. Doxorubicin, as well as alkylating agents, generate reactive oxygen species and radical-containing molecules. This represents another explanation for the predictive power of doxorubicin towards alkylating drugs.

Broad spectrum resistance represents a severe problem in the clinic, and combination regimen frequently fails to cure cancer patients from their disease. This alarming situation requires the development of novel diagnostic means as well as novel treatment options to improve treatment success.

An attractive diagnostic approach delineated from the results shown in the present investigation represents the prediction of individual response of a cancer patient to therapy. Although the statistical probability of therapeutic success is well-known for larger groups of patients from clinical therapy trials, it is, however, still not possible to predict which individual cancer patient will respond to chemotherapy. It would be, therefore, of great value for patients to know whether or not a tumor would respond to the proposed therapy or whether undesirable side effects may occur (21, 22). Then, the optimal treatment schedule could be adapted for each individual patient. This concept of individualized tumor therapy is not only valuable for established anticancer drugs such as doxorubicin but is also of great importance for other small molecule inhibitors derived from TCM.

In an endeavor to improve cancer treatment, the concept of the blockage of P-glycoprotein by specific inhibitors has been devised during the past years. Despite huge efforts in the academia and industry, no P-glycoprotein inhibitor has yet reached the pharmaceutical market (23, 24).

Another strategy is the development of specific inhibitors for novel tumor-associated target proteins. Prominent examples are inhibitors of tyrosine kinases (i.e., imatinib mesylate, Gleevec). Despite impressive activity in the clinical setting, those compounds are also prone to the development of resistance (25, 26).

A third strategy represents the identification of cytotoxic compounds from natural resources which are not recognized by classical mechanisms such as P-glycoprotein and which reveal pleiotropic modes of action. Synthetic tyrosine kinase inhibitors such as imatinib mesylate (Gleevec) act specifically towards few targets (i.e., bcr-abl or KIT; refs. 25, 26). Point mutations in these target proteins diminish the binding affinities of these compounds and render tumor cells resistant. In contrast, many natural products have several targets. For instance, secondary metabolites of plants have been developed during evolution to protect against microbial attack (bacteria, viruses), against parasites (insects, worms), and against herbivores. Molecules with pleiotropic actions may, therefore, be more efficient for plants than monospecific ones (27). Novel developments of chemically synthesized small molecules take this into account and attack several target molecules in cancer cells. Sunitinib acts in an antiproliferative and antiangiogenic manner and inhibits all vascular endothelial growth factor receptor subtypes and platelet-derived growth factor receptors (28). The multikinase inhibitor sorafenib was developed as an inhibitor of RAF-1, but turned out to inhibit subtypes of the vascular endothelial growth factor receptor family, the platelet-derived growth factor receptor, Flt-3, and c-kit as well (29).

This was the starting point for us to screen for cytotoxic compounds from plants with activity against tumor cells. We focused on medicinal plants used in TCM (30, 31). The 5,000-year-old tradition of TCM resulted in a pharmacopoeia with highly active and efficient recipes. Therefore, it can be expected that the search for novel cytotoxic compounds may be more promising than a blind screening of plants.

Recently, we have established a database with >2,400 chemically characterized compounds, of which 531 compounds exerted considerable cytotoxicity against tumor cells (18). As shown in the present investigation, a major fraction of these compounds derived from TCM act independently of the proliferative activity of tumor cells and are not affected by P-glycoprotein–mediated efflux. This gives reason to hope that TCM represents a valuable reservoir for novel anticancer agents with activity against otherwise drug-resistant tumors.

The identification of non–cross-resistant compounds from TCM do not imply that these natural products are at the same time inhibitors of P-glycoprotein. P-glycoprotein inhibitors could not be identified by our database approach. Rather it is necessary to perform combination treatment experiments, i.e., by combining doxorubicin with a natural product to see whether there is an overadditive or synergistic cytotoxic effect compared with both compounds alone. In an ongoing project, we started the search for novel P-glycoprotein inhibitors from natural products from TCM. Four geranylated furocoumarins from the fruits of *Tetradium daniellii* (Rutaceae) were tested in a bioassay using CCRF-CEM leukemia cells and their P-glycoprotein–overexpressing subline, CEM/ADR5000, to assess their cytotoxicity and effects on modulation of P-glycoprotein function. All four substances showed considerable proliferation inhibition. An assay monitoring the P-glycoprotein–dependent accumulation of a calcein fluorescent dye showed that these four substances also inhibited P-glycoprotein function (32). In another project, the quinolones 1-methyl-2-undecyl-4-quinolone, dihydroevocarpine, and evocarpine, as well as the indoloquinazoline alkaloids, rutacarcpine and evodiamine—all from the Chinese medicinal herb *Evodia rutaecarpa* (Rutaceae) were analyzed. Although all substances inhibited cell growth, only the 1-methyl-2-undecyl-4-quinolone and dihydroevocarpine were also modulators of P-glycoprotein activity (33). We will systematically exploit the chances to develop novel cancer drugs from TCM with improved features in the years to come.

References

1. Rhoads CP. Report on a cooperative study of nitrogen mustard (HN2) therapy of neoplastic disease. Trans Assoc Am Physicians 1947;60:110–7.
2. Cortazar P, Johnson BE. Review of the efficacy of individualized chemotherapy selected by *in vitro* drug sensitivity testing for patients with cancer. J Clin Oncol 1999;17:1625–31.
3. Salmon SE, Hamburger AW, Soehnlein B, Durie BG, Alberts DS, Moon TE. Quantitation of differential sensitivity of human tumor stem cells to anticancer drugs. N Engl J Med 1978;298:1321–7.
4. Volm M, Kaufmann M, Hinderer H, Goerttler K. Rapid method for sensitivity testing of tumors against cytostatic agents. Klin Wochenschr 1970;48:374–6.
5. Volm M, Wayss K, Kaufmann M, Mattern J. Pretherapeutic detection of tumour resistance and the results of tumour chemotherapy. Eur J Cancer 1979;15:983–93.
6. Volm M, Drings P, Hahn EW, Mattern J. Prediction of the clinical chemotherapeutic response of the stage III lung adenocarcinoma patients by an *in vitro* short term test. Br J Cancer 1988;57:198–200.

7. Group of Sensitivity Testing of Tumors (KSST): *in vitro* short term test to determine the resistance of human tumours to chemotherapy. Cancer 1981;48: 2127–35.

8. Kimmig A, Gekeler V, Neumann M, et al. Susceptibility of multidrug-resistant human leukemia cell lines to human interleukin 2-activated killer cells. Cancer Res 1990;50:6793–9.

9. Gillet JP, Efferth T, Steinbach D, et al. Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. Cancer Res 2004;64:8987–93.

10. Wang YF, Cao JX, Efferth T, Lai GF, Luo SD. Cytotoxic and new tetralone derivatives from *Berchemia floribunda* (Wall.) Brongn. Chem Biodivers 2006;3:646–53.

11. Wang YF, Lai GF, Efferth T, Cao JX, Luo SD. New glycosides from *Tetracentron sinense* and their cytotoxic activity. Chem Biodivers 2006;3:1023–30.

12. Kaspers GJ, Pieters R, van Zantwijk CH, et al. *In vitro* drug sensitivity of normal peripheral blood lymphocytes and childhood leukaemic cells from bone marrow and peripheral blood. Br J Cancer 1991;64:469–74.

13. Rubinstein LV, Shoemaker RH, Paull KD, et al. 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 1990;82:1113–8.

14. Alley MC, Scuderio DA, Monks A, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988;48:589–601.

15. Wosikowski K, Schuurhuis D, Johnson K, et al. Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. J Natl Cancer Inst 1997;89: 1505–15.

16. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24:236–44.

17. Staunton JE, Slonim DK, Coller HA, et al. Chemosensitivity prediction by transcriptional profiling. Proc Natl Acad Sci U S A 2001;98:10787–92.

18. Konkimalla VB, Efferth T. Anti-cancer natural product library from traditional Chinese medicine. Comb Chem High Throughput Screen 2008;11:7–15.

19. Efferth T. The human ATP-binding cassette transporter genes: from the bench to the bedside. Curr Mol Med 2001;1:45–65.

20. Gillet JP, Efferth T, Remacle J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. Biochem Biophys Acta Rev Cancer 2007;1775:237–62.

21. Volm M, Koomägi R, Efferth T. Prediction of drug sensitivity and resistance of cancer by protein expression profiling. Cancer Genomics Proteomics 2004;1: 157–66.

22. Efferth T, Volm M. Pharmacogenetics and individualized cancer chemotherapy. Pharmacol Ther 2005; 107:155–76.

23. Nobilit S, Landini I, Giglioni B, Mini E. Pharmacological strategies for overcoming multidrug resistance. Curr Drug Targets 2006;7:861–79.

24. Takara K, Sakaeda T, Okumura K. An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. Curr Pharm Des 2006;12: 273–86.

25. Bickenbach K, Wilcox R, Veerapong J, et al. A review of resistance patterns and phenotypic changes in gastrointestinal stromal tumors following imatinib mesylate therapy. J Gastrointest Surg 2007;11:758–66.

26. Hochhaus A, Erben P, Ernst T, Mueller MC. Resistance to targeted therapy in chronic myelogenous leukemia. Semin Hematol 2007;44:S15–24.

27. Wink M, Plant breeding: Importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet 1988;75: 225–233.

28. Chow LQ, Eckhardt SG. Sunitinib: from rational design to clinical efficacy. J Clin Oncol 2007;25: 884–96.

29. Wilhelm S, Carter C, Lynch M, et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Nat Rev Drug Discov 2006; 5:835–44.

30. Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR. The anti-malarial artesunate is also active against cancer. Int J Oncol 2001;18:767–73.

31. Efferth T, Davey M, Olbrich A, Rücker G, Gebhart E, Davey R. 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 2002;28:160–8.

32. Adams M, Mahringer A, Bauer R, Fricker G, Efferth T. *In vitro* cytotoxicity and P-glycoprotein modulating effects of geranylated furocoumarins from *Tetradium daniellii*. Planta Med 2007;73:1475–8.

33. Adams M, Mahringer A, Kunert O, Fricker G, Efferth T, Bauer R. Cytotoxicity and P-glycoprotein modulating effects of quinolones and indoquinazolines from the Chinese herb *Evodia rutaecarpa*. Planta Med 2007;73:1554–7.