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mRNA expression profiles for the response of human tumor cell lines to the antimalarial drugs artesunate, arteether, and artemether

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Abstract

The antimalarial artemisinin derivatives artesunate (ART), arteether (ARE), and artemether (ARM) reveal remarkable antineoplastic activity. In the present investigation, we identified mRNA expression profiles associated with the response of tumor cells to ART, ARE, and ARM. We performed correlation and hierarchical cluster analyses of inhibition concentration 50% (IC_{50}) values and basal mRNA expression levels of 464 genes deposited in the database of the National Cancer Institute, USA. Correlating IC_{50} values of ART, ARE, and ARM and of 16 established antineoplastic drugs revealed that the artemisinin derivatives could not be assigned with a known class of drugs with defined mode(s) of action. The basal mRNA expression of 208 out of 464 genes (45%) correlated significantly with IC_{50} values of at least one artemisinin derivative. These genes were from different classes (drug resistance genes, DNA damage and repair genes, apoptosis-regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes and cytokines). We identified two different gene clusters by hierarchical cluster analysis. One cluster contained predominately genes significantly correlated to all three artemisinin derivatives. This overlapping set of genes points to common molecular mechanisms of tumor inhibition by all three drugs in which genes affecting cellular proliferation may play an important role. The second cluster contained genes differentially associated with the response of artemisinin derivatives to cancer cells. The number of correlating drug resistance genes in this cluster increased in the order ART < ARE < ARM and was paralleled by increasing IC_{50} values of the three drugs in the same order. The higher activity of ART in comparison to ARE and ARM may, thus, be explained by a lower number of drug resistance genes affecting ARTs action. The present analysis is a starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional role of individual genes for the activity of artemisinin derivatives in tumor cells.

Keywords: Chemotherapy; Microarray analysis; Hierarchical cluster analysis

1. Introduction

Artemisia annua L. (*qinhao*) has been used for centuries in traditional Chinese medicine (TCM) to treat fever and malaria. Its description dates back to 340 B.C. in ''The Handbook of Prescriptions for Emergencies'' from Ge Hong, East Jin Dynasty [1]. The active principle of *qinhao* has been identified by Chinese scientists in the early 1970s and was named *qinhaosu* (artemisinin) [2]. Artemisinin exerts remarkable activity against otherwise drug-resistant *Plasmodium falciparum* and *Plasmodium vivax* strains and is, thus, gaining increasing importance in the treatment of unresponsive malaria infections. In the past years, semi-synthetic derivatives of artemisinin have been developed (artesunate, arteether, artemether). The World Health Organization recommends artemisinin derivatives as emergency drugs in regions with resistant *Plasmodium* strains, e.g. Southeast Asia and Western Africa. Though the chemical modifications are not in the reactive endoperoxide moiety, these derivatives differ in their antimalarial properties [3]. Apart from their inhibitory activity against *Plasmodium*, artemisinin derivatives exert profound activity against tumor cells [4–6]. Through the formation of radical molecules and/or reactive oxygen species is common to artemisinin drugs and contributes to their antimalarial activity [7,8], it is not known which genetic pathways are involved in cancer cells and to which extent they vary in different derivatives.

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Abbreviations: ARE, arteether; ARM, artemether; ART, artesunate.

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Under the auspices of the National Cancer Institute, USA, we analyzed artesunate in 55 cell lines of different tumor types [5]. The aim of the present investigation was to identify genes associated with the response of tumor cells to ART, ARE, and ARM. For this reason, we correlated the inhibition concentration 50% (IC_{50}) values for these artemisinin derivatives with the basal mRNA expression levels of 464 genes obtained by microarray assays [9].

2. Material and methods

2.1. Cell lines of the anticancer drug screen

The origin and processing of the cell lines of the Developmental Therapeutics Program of the National Cancer Institute of the USA were previously described [10]. The panel for the present investigations consisted of 55 human tumor cell lines representing leukemia (CCRF-CEM, HL-60, K-562, MOLT-4, RPMI-8226, SR), melanoma (LOX IMVI, M14, SL-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257, UACC-62), non-small cell lung cancer (A549, EKVX, HOP-62, HOP-92, NCI-H322M, NCI-460), colon cancer (COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12, SW-620), renal cancer (786-0, A498, ACHN, CAKI-1, SN12C, TK-10, UO-31), ovarian carcinoma (IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3), tumors of the central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251), prostate carcinoma (PC-3, DU-145), and breast cancer (MCF-7, NCI-ADR Res, MDA-MB-231, HS578T, MDA-MB-435, MDA-N, BT-549, T-47D).

2.2. Statistical analyses

Hierarchical cluster analysis is an explorative statistical method and aims to group at first sight heterogeneous objects into clusters of homogeneous objects. Objects are classified by calculation of distances according to the closeness of between-individual distances. All objects are assembled into a cluster tree (dendrogram). The merging of objects with similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relatedness. The procedure continues to aggregate clusters until there is only one. The distance of 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, while the separation in the cluster tree increases with progressive dissimilarity. Cluster analyses applying the complete-linkage method were done by means of the WinSTAT program (Kalmia Co.). 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 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. To visualize the relationships between the IC_{50} values for ART, ARM, or ARE and mRNA expression levels by cluster analyses, cluster image maps were formed.

2.3. Kendall's τ-test

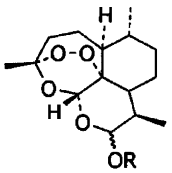
For calculation of the correlation of ART to other drugs as well as to mRNA expression, we used Kendall's τ-rank correlation test. This test was implemented into the WinSTAT program (Kalmia Co.). Rank correlation was calculated to obtain a relative measure for the linear dependence of two variables. Kendall's τ-test determines the correlation of rank positions of values. Ordinal or metric scaling is suited for the test and are transformed in rank positions. There is no conditions regarding distribution forms, e.g. normal distribution, for the performance of Kendall's τ-test.

2.4. COMPARE analysis

COMPARE analyses were performed with a software implemented in the web site of the National Cancer Institute, USA (http://dtp.nci.nih.gov). COMPARE analyses are rank-ordered lists of compounds. Every compound of the Standard Agent Database of the National Cancer Institute is ranked for similarity of its *in vitro* cell growth pattern to the *in vitro* cell growth pattern of a selected seed or probe compound [11]. To derive COMPARE rankings, a scaler index of similarity between the seed compound cell growth pattern and the pattern for each of the COMPARE database compounds is created. This methodology has been exploited to identify the presumable mode of action of investigational drugs by comparing their IC_{50} profiles of the NCI cell lines with those of drugs with well characterized mechanisms of action [12].

3. Results

ART, ARE, and ARM (Fig. 1) have been tested over a dose range from 10^{-8} to 10^{-4} M in 55 cell lines of the National Cancer Institute's screening panel and IC_{50} values had been calculated thereof ([5], http://dtp.nci.nih.gov). The IC_{50} values of the 55 cell lines for the three drugs are shown in Fig. 2a. A comparison of the IC_{50} values showed



R = CH_3: Artemether

R = C_2H_5: Arteether

R = $C-CH_2-CH_2-COONa$ (O)

Artesunate

Fig. 1. Chemical structure of ART, ARE, and ARM.

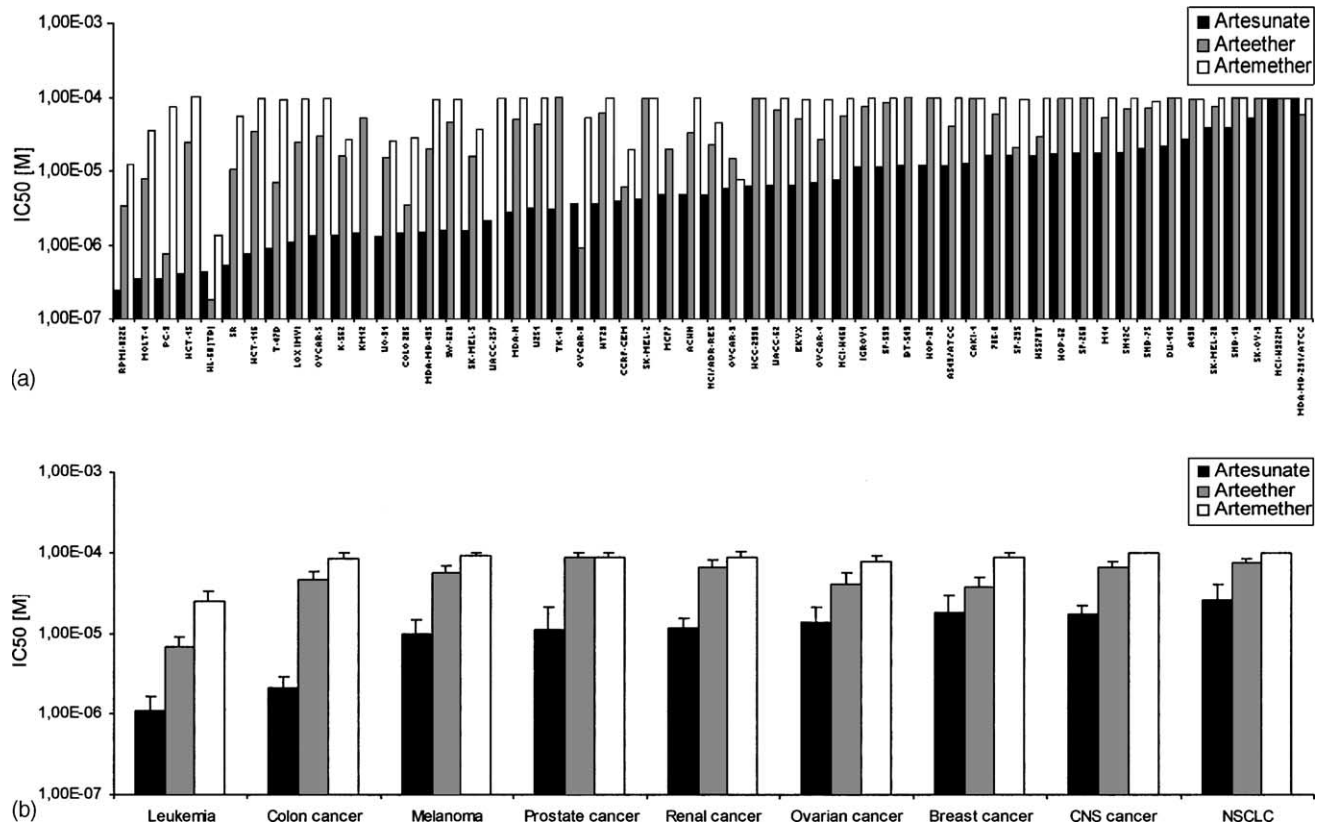


Fig. 2. Inhibition concentration 50% (IC_{50}) values of artemisinin derivatives (ART, ARE, ARM) calculated from dose–response curves. (a) The IC_{50} values of 55 cell lines and (b) mean values and SEM of IC_{50} values of 55 cell lines grouped according to their tumor origin.

that ART was the most active drug followed by ARE and ARM (Fig. 2). The overall mean IC_{50} values of all 55 cell lines was 12.3 μM (ART), 49.6 μM (ARE), and 82.4 μM (ARM), respectively. Leukemia cell lines were most sensitive towards all three drugs, while non-small cell lung cancer (NSCLC) cell lines were most resistant (Fig. 2b).

The IC_{50} values of the three drugs of all 55 cell lines were subjected to Kendall's τ-test. As shown in Table 1, the relationships between the three artemisinin derivatives were highly significant indicating that the cell lines responded similarly towards the three derivatives. Then, the IC_{50} values of the 55 cell lines for ART, ARM, and ARE were correlated with those for established drugs. The *P*-values obtained by Kendall's τ-test are shown in Table 2. ART, ARM, and ARE correlated significantly with some of the drugs investigated, e.g. BCNU, CCNU, tetraplatin, paclitaxel, and 6-mercaptopurine, but not to others, e.g. ifosfamide, cisplatin, camptothecin, topotecan, doxorubicin, etoposide, mAMSA, 5-fluorouracil, and cytosine–arabinoside.

Table 1
Correlation of the IC_{50} values for artemisinin derivatives in 55 human tumor cell lines of the NCI screening panel

	ART	ARM
ARM	2.241^{e-04} [a]	–
ARE	1.050^{e-09}	5.011^{e-07}

[a] *P*-value (Kendall's τ-test).

Table 2
Correlation of the IC_{50} values for the artemisinin derivatives ART, ARE, and ARM with those for established antineoplastic agents in 55 human tumor cell lines of the NCI screening panel

	ART	ARE	ARM
Alkylating agents			
Carmustine (BCNU)	0.00481[a]	0.01013	0.01828
Lomustine (CCNU)	0.01748	0.0322	0.04436
Ifosfamide	n.s.	n.s.	n.s.
Melphalan	n.s.	0.01958	n.s.
Platinum compounds			
Cisplatin	n.s.	n.s.	n.s.
Tetraplatin	0.00018	0.00005	0.00133
Tubulin inhibitors			
Paclitaxel	0.04568	0.02427	0.03834
Vincristine	n.s.	0.00438	n.s.
Topoisomerase I poisons			
Camptothecin	n.s.	n.s.	n.s.
Topotecan	n.s.	n.s.	n.s.
Topoisomerase II poisons			
Doxorubicin	n.s.	n.s.	n.s.
Etoposide	n.s.	n.s.	n.s.
mAMSA	n.s.	n.s.	n.s.
Antimetabolites			
5-Fluorouracil	n.s.	n.s.	n.s.
Cytosine–arabinoside	n.s.	n.s.	n.s.
6-Mercaptopurine	0.00853	0.02031	0.00874

Not significant, n.s. ($P > 0.05$).
[a] *P*-value (Kendall's τ-test).

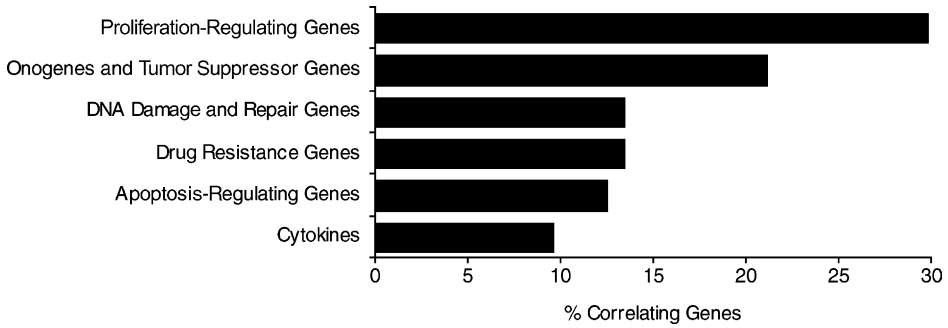


Fig. 3. Percentage of genes belonging to six functional categories, whose basal mRNA expression in 55 cell lines correlated with the IC_{50} values for ART, ARE, and ARM. Expression of mRNA has been determined by microarray analysis [9] and deposited in the database of the NCI (http://dtp.nci.nih.gov). Expression of mRNA was given in relative units as log (mRNA levels in cell line/mRNA levels in reference pool). Reference probes were made by pooling equal amounts of mRNA from HL-60, K-562, NCI-H226, COLO 205, SNB-19, LOX-IMVI, OVCAR-3, OVCAR-4, CAKI-1, PC-3, MCF-7, and HS568T cell lines.

These data were further subjected to COMPARE analysis. The COMPARE computations of ART, ARE, and ARM against 171 agents included in the Standard Agent Database of the NCI did not reveal any correlation coefficients at the IC_{50} level exceeding 0.6 (data not shown). This implies that no information can be gained from COMPARE analysis with regard to the mode of action of ART. Thus, ART does likely not belong to the traditional classes of antitumor drugs (i.e. topoisomerase I/II inhibitors, tubulin poisons, DNA alkylators, DNA/RNA intercalators, etc.).

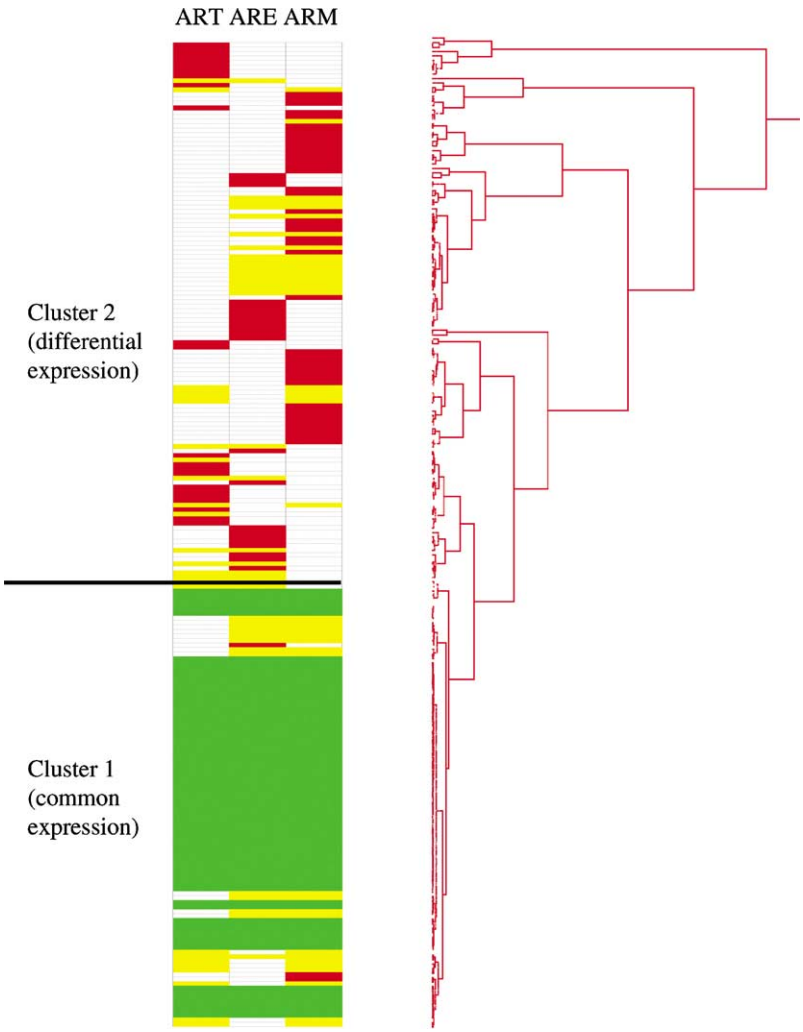


Fig. 4. Hierarchical cluster analysis (complete-linkage analysis) of *P*-values obtained by Kendall's τ-test of significantly correlating mRNA gene expressions with artemisinin derivatives.

Therefore, we searched for molecular determinants of ARTs, AREs, and ARMs action against tumor cells.

Next, we correlated the IC_{50} values of ART, ARE, and ARM with the basal mRNA expression levels of 464 genes of the 55 cell lines by means of Kendall's τ-test. These genes were selected from gene categories which are frequently involved in the responsiveness of established anticancer drugs (drug resistance genes, DNA damage and repair genes, apoptosis-regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes, and cytokines) assuming that those genes may also be relevant for the activity of artemisinin derivatives. Of these 464 genes, 208 correlated significantly with ART, ARE, or ARM (45%). As can be seen in Fig. 3, the portions of proliferation-regulating genes and oncogenes/tumor suppressor genes, whose expression correlated with the IC_{50} values for ART, ARE, and ARM, are considerably higher that of the other gene classes.

The *P*-values of these 208 genes were subjected to hierarchical cluster analysis. In Fig. 4, each value was color-coded: green was used, if *P* was <0.05 for all three artemisinin derivatives. Yellow indicated $P < 0.05$ for two and red for one of the drugs. Two major clusters were observed. Cluster 1 contained genes whose expression correlated with one or two of the artemisinin derivatives only. A second cluster consisted predominately of gene expressions commonly associated with all three compounds. The distribution of genes of the different categories (drug resistance genes, DNA damage and repair genes, apoptosis-regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes, and cytokines) was not different between both clusters (Fig. 5).

Therefore, we focused on genes of cluster 1 which were differentially associated with the three drugs and found that the number of drug resistance genes varied between the three artemisinin derivatives. As shown in Table 3, ART,

Table 3
Correlation of the IC_{50} values for artemisinin derivatives with basal mRNA expression of drug resistance genes in 55 human tumor cell lines of the NCI screening panel

Gene symbol[a]	Gene name	ART	ARE	ARM
CAT	Catalase	0.03857[b]	0.01075	0.00310
GLCLR	γ-Glutamylcysteine synthetase	0.00046	0.00558	0.04747
GSTZ1	Glutathione *S*-transferase Z1	0.02174	0.02451	0.03179
TOP1	DNA topoisomerase I	0.00004	0.00008	0.00148
TXNPOX	Thioredoxin peroxidase	0.04214	0.04383	0.03621
TXNRD1	Thioredoxin reductase 1	0.00972	0.0107	0.03119
AOP2	Antioxidative protein 2	0.02321	0.04896	n.s.
MGST3	Mitochondrial glutathione *S*-transferase 3	0.00367	0.04387	n.s.
GSTM4	Glutathione *S*-transferase M4	0.03097	n.s.	0.00709
GSTT2	Glutathione *S*-transferase T2	0.00218	n.s.	0.00444
TOP2B	DNA topoisomerase II β	0.02396	0.00276	n.s.
ALDH5A1	Aldehyde dehydrogenase 5A1	n.s.	0.02279	0.00292
ALDH6	Aldehyde dehydrogenase 6	n.s.	0.02912	0.00072
GPX4	Glutathione perodidase 4	n.s.	0.0197	0.03174
MT1E	Metallothioneine 1E	n.s.	0.03093	0.01398
MT1H	Metallothionein 1H	n.s.	0.00191	0.00149
MT1L	Metallothionein 1L	n.s.	0.02932	0.00410
MT2A	Metallothionein 2A	n.s.	0.00579	0.00135
GPX1	Glutathione peroxidase 1	n.s.	0.03142	n.s
GSTA2	Glutathione *S*-transferase A2	n.s.	0.01149	n.s.
HPRT1	Hypoxanthine phosphoribosyltransferase 1	n.s.	0.00634	n.s.
ALDH1	Aldehyde dehydrogenase 1	n.s.	n.s.	0.02195
ALPP	Alkaline phosphatase	n.s.	n.s.	0.00539
DIA4	Diaphorase (NADH/NADPH) cytochrome *b*-5 reductase	n.s.	n.s.	0.03019
DHFR	Dihydrofolate reductase	n.s.	n.s.	0.00942
GSTM3	Glutathione *S*-transferase M3	n.s.	n.s.	0.02019
OSR1	Oxidative stress response 1	n.s.	n.s.	0.00107
RRM1	Ribonucleotide reductase M1 polypeptide	n.s.	n.s.	0.00171
RRM2	Ribonucleotide reductase M2 polypeptide	n.s.	n.s.	0.04841
Total number		11	18	24

Not significant, n.s. ($P > 0.05$).
[a] Human Genome Organization approved gene symbols as used by the National Cancer Institute, USA (http://dtp.nci.nih.gov).
[b] *P*-value (Kendall's τ-test).

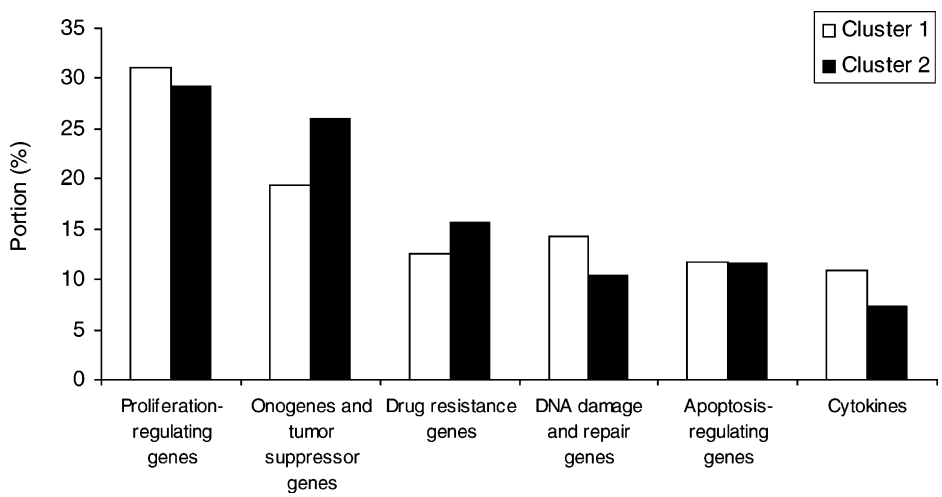


Fig. 5. Percentages of genes of different gene classes in the two clusters shown in Fig. 4.

ARE, and ARM correlated with the expression of 11, 18, and 24 drug resistance genes, respectively. There was a relationship between the mean IC_{50} values of ART, ARE, and ARM for all 55 cell lines and the number of correlating genes ($R^2 = 0.999$; $P = 0.058$).

4. Discussion

The antimalarial artemisinin derivatives ART, ARE, and ARM also possess antineoplastic activity as shown by us and others [4–6]. In the present investigation, we tried to get insight into the molecular pharmacology of their antineoplastic features. First, we analyzed the cross-resistance pattern of ART, ARE, and ARM to 16 standard antineoplastic agents of different drug classes (alkylating agents, platinum compounds, tubulin inhibitors, topoisomerase I or II poisons, and antimetabolites) by Kendall's τ-test. Then, we extended these analysis using COMPARE statistics and 171 drugs from the Standard Agent Database of the NCI. The fact that ART, ARM, and ARE could not be assigned to one of these drug classes is a clue that the artemisinin derivatives may not belong to one of these classes of cytostatic drugs with known mode(s) of action.

The highest *in vitro* activity was found with ART as compared to ARM and ARE. This was in consistency with the reported antimalarial activities *in vitro* for these three drugs [3].

Through the formation of radical molecules and/or reactive oxygen species is common to artemisinin drugs and contributes to their antimalarial activity, it is not known which genetic pathways are involved in cancer cells and to which extent they vary in different derivatives. The response of tumors towards chemotherapy can be predicted by microarray-based gene expression profiling approaches as previously shown for other cytostatic drugs [7,13]. For this reason, we analyzed the relationship of artemisinin derivatives to the basal mRNA expression profiles of 464 genes. These genes were taken from classes which are frequently involved in the response of tumor cells to established antineoplastic drugs (drug resistance genes, DNA damage and repair genes, apoptosis-regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes, and cytokines). We assumed that these gene classes may also be relevant for artemisinin derivatives. Two different gene clusters became apparent by means of hierarchical cluster analysis. One cluster contained predominately genes significantly correlated to all three artemisinin derivatives. This overlapping set of genes points to common molecular mechanisms of tumor inhibition by all three drugs in which genes affecting cellular proliferation may play an important role. The significant correlation of IC_{50} values of ART, ARE, and ARM to each other is consistent with this finding. The second cluster contained genes differentially associated with the response of artemisinin derivatives to cancer cells. The number of correlating drug resistance genes in this cluster increased in the order ART < ARE < ARM and was paralleled by increasing IC_{50} values of the three drugs in the same order. The expression of several drug resistance genes correlated with the IC_{50} values of ART, ARE, and ARM, especially genes which are involved in the detoxification of electrophilic compounds (glutathione-associated enzymes, *AOP2*, catalase, thioredoxin peroxidase and reductase, OSR1). This is conceivable with the reaction of the endoperoxide bridge of ART to form radical molecules which play an important role for the antimalarial activity of ART [14]. Once inside the parasites within erythrocytes, the endoperoxide bridge of ART is opened initiating oxidative reactions. These produce reactive oxygen species and/or ART carbon-centered free radicals both of which affect cellular proteins and lipids of the parasites [7,8]. The opening of the endoperoxide bridge is facilitated by free Fe^{2+} ions and heme-bound iron from the blood. A role of the endoperoxide bridge can also be assumed for the antineoplastic activity. The higher activity of ART in comparison to ARE and ARM may, therefore, be explained by a lower number of drug resistance genes affecting ARTs action.

In summary, the present investigation showed that the anticancer activity of ART, ARE, and ARM is associated with the basal mRNA expression of genes of different gene classes. Genes which affect the proliferation of cells (cell cycle regulating genes, growth factors and their receptors, oncogenes, tumor suppressor genes) may play an important role, although this hypothesis has yet to be confirmed in further investigations. The large number of genes involved can be reconciled with previous findings on the multifactorial nature of chemosensitivity [15–18]. The present analysis may be a valuable starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional role of individual genes for the activity of artemisinin derivatives in tumor cells.

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