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Cytotoxic activity of secondary metabolites derived from *Artemisia annua* L. towards cancer cells in comparison to its designated active constituent artemisinin

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ABSTRACT

Artemisia annua L. (sweet wormwood, *qinhao*) has traditionally been used in Chinese medicine. The isolation of artemisinin from *Artemisia annua* and its worldwide accepted application in malaria therapy is one of the showcase success stories of phytomedicine during the past decades. Artemisinin-type compounds are also active towards other protozoal or viral diseases as well as cancer cells *in vitro* and *in vivo*. Nowadays, *Artemisia annua* tea is used as a self-reliant treatment in developing countries. The unsupervised use of *Artemisia annua* tea has been criticized to foster the development of artemisinin resistance in malaria and cancer due to insufficient artemisinin amounts in the plant as compared to standardized tablets with isolated artemisinin or semisynthetic artemisinin derivatives. However, artemisinin is not the only bioactive compound in *Artemisia annua*. In the present investigation, we analyzed different *Artemisia annua* extracts. Dichloromethane extracts were more cytotoxic (range of IC_{50}: 1.8–14.4 μg/ml) than methanol extracts towards *Trypanosoma b. brucei* (TC221 cells). The range of IC_{50} values for HeLa cancer cells was 54.1–275.5 μg/ml for dichloromethane extracts and 276.3–1540.8 μg/ml for methanol extracts. Cancer and trypanosomal cells did not reveal cross-resistance among other compounds of *Artemisia annua*, namely the artemisinin-related artemisitene and arteanuine B as well as the unrelated compounds, scopoletin and 1,8-cineole. This indicates that cells resistant to one compound retained sensitivity to another one. These results were also supported by microarray-based mRNA expression profiling showing that molecular determinants of sensitivity and resistance were different between artemisinin and the other phytochemicals investigated.

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Introduction

Artemisia annua L. (sweet wormwood, *qinghao*) has traditionally been used in China for the treatment of fever and chills. Artemisinin has been identified as the anti-malarial principle of the plant, and artemisinin derivatives are nowadays established as anti-malarial drugs with activity towards otherwise drug-resistant *Plasmodium* infections. Though originally growing in Asia and Europe, the plant is cultivated in Africa and used as tea for the treatment of malaria. Artemisinin-type compounds are not only active towards malaria, but also towards a variety of other diseases such as infections with *Schistosoma*, *Leishmania*, *Trypanosoma*, a wide variety of viruses and human cancer cell lines *in vitro* and *in vivo*, and even plant crown gall tumors (Efferth et al. 2003, 2008; Efferth 2005, 2007, 2009; Dell'Eva et al. 2004; Ullrich et al. 2009; Nibret and Wink 2010).

Despite artemisinin's global application in malaria therapy is one of the showcase success stories of pharmacognosy during the past decades, there are still some contradictions which have not satisfactorily been addressed as yet. Artemisinin is not or hardly water soluble, but the traditional use in Chinese medicine is based on water preparations such as tea or decoction. Nowadays, *Artemisia annua* tea is used as a self-reliant treatment for malaria in developing countries (Mueller et al. 2000; de Ridder et al. 2008; RITAM Artemisia annua Task Force 2006). Especially the unsupervised use of *Artemisia annua* tea has been criticized. It has been argued that the use of suboptimal concentrations of artemisinin would facilitate the development of resistance (Jansen 2006). It is, however, a general biological phenomenon that medicinal plants contain rather many than single pharmacologically active phytochemical compounds. This is well-known and thoroughly discussed for many years as one of the advantages of phytotherapy (including traditional Chinese medicine) compared to classical Western medicine (Wink 2008). In *Artemisia annua*, more than 50 different phytochemicals have been recorded (Dr. Duke's Phytochemical and Ethnobotanical Databases; http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl). It can, hence, be hypothesized

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that the development of resistance, which is not recorded for the use of this plant in traditional Chinese medicine, might not take place. Rather the plant provides a sort of combination therapy which engraves or even prevents the development of resistance to single bioactive plant constituents. The bioactivity of other constituents of *Artemisia annua* apart from artemisinin has, however, not adequately been addressed as yet.

In an effort to evaluate, whether other constituents than artemisinin in *Artemisia annua* may also reveal cytotoxicity towards cancer cells we focused on two artemisinin-related compounds, arteanuine B and artemisitene, and two other compounds without structural similarity to artemisinin, scopoletin and 1,8-cineole, which are also present in this plant. First, we analyzed the inhibitory activity of these compounds towards human HeLa cervical cancer cells. To prove a broader bioactivity in addition to cytotoxicity towards cancer cells, the inhibitory action towards *Trypanosoma* has been investigated. As a second step, we analyzed cross-resistance profiles of these compounds in the NCI panel of cell lines derived from different tumor types. Microarray-based mRNA expression profiling and COMPARE analyses revealed that different sets of genes correlated with the IC_{50} values for these compounds, indicating that the missing cross-resistance of arteanuine B, artemisitene, 1,8-cineole or scopoletin towards artemisinin may be based on different transcriptomic expression profiles determining sensitivity or resistance to these compounds.

Materials and methods

Plant material

Artemisia annua was obtained from different sources to test the variability of different specimens. *Artemisia annua* specimen A was obtained from a pharmacy in Germany. The origin of this specimen was China, the harvest date is unknown. *Artemisia annua* specimen B was grown in Tansania/Bunda and was obtained from the non-governmental organisation, Anamed (pulverized leaves; harvest date: 2008). Further specimens were also provided by Anamed: specimen C (grown in Winnenden, Germany, screen leftovers and thin caulis, harvest date: 2007) and specimen D (Winnenden, chaff and thick caulis, harvest date: 2007). A further specimen was purchased on a medicinal plant market in Shanghai, China (specimen E; harvest date: 2005).

These plant samples were macerated in dichloromethane or methanol, and left on a shaker for two days. The extracts were filtered and evaporated to dryness under reduced pressure using Rotavapor as described (Nibret and Wink 2010).

Phytochemicals

Artemisinin was obtained from Sigma–Aldrich (Taufkirchen, Germany) and artesunate was purchased from Saokim Ltd. (Hanoi, Vietnam). Arteanuine B and artemisitene were obtained from the drug repository of the Developmental Therapeutics Program of the National Cancer Institute (NCI, Bethesda, MA, USA). Scopoletin and 1,8-cineole were purchased from Sigmal–Aldrich (Taufkirchen, Germany). The chemical structures of the compounds are depicted in Fig. 1.

Determination of plant constituents by GLC–MS

The analysis was carried out on a Hewlett-Packard gas chromatograph (GC 5890II, Hewlett PACKARD; Bad Homburg, Germany) equipped with OV-1 column (30 m × 0.25 mm × 0.25 μm; Ohio Valleys, Ohio, USA). The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ7000, Thermo-Finnigan, Bremen, Germany). The operation conditions were previously described (Nibret and Wink 2010).

Cell lines

TC221 *Trypanosoma brucei brucei* cells, the causative agent of Nagana epidemic, were grown in Baltz medium supplemented with 20% inactivated fetal bovine serum (FBS) and 0.001% β-mercaptoethanol as previously described (Baltz et al. 1985).

Human HeLa cervical carcinoma cells were cultured in DMEM complete medium supplemented with 10% inactivated FBS, 1% antibiotics (penicillin, streptomycin) and 1% NEA. Cells were maintained in a humified atmosphere containing 5% CO_2 at 37 °C.

The panel of human tumor cell lines of the Developmental Therapeutics Program of NCI consisted of leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian cancer cells, tumor cells of the central nervous system, prostate carcinoma, and breast cancer. Their origin and processing have previously been described (Alley et al. 1988). These cell lines were employed to determine the cytotoxicity of the isolated compounds and of topotecan, irinotecan and SN-38 as positive controls.

Cytotoxicity assays

Sensitivity of TC221 and HeLa cells towards extracts and pure compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Mosmann 1983). 1×10^5 cells/ml were seeded in 96 well plates and cultured for 24 h. They were treated with 0–5 mg/ml of *Artemisia annua* extract 0–5 mg/ml pure compound for 24 h. Medium was exchanged with fresh medium containing 1 mg/ml MTT and incubated for 4 h. The formazan crystals were dissolved in 100 μl DMSO. The absorbance was measured at 570 nm with a Tecan Safire II Reader (Tecan, Männedorf, Switzerland). All experiments were performed in triplicates and repeated three times. The viability results of TC221 cells were additionally confirmed by counting under the microscope. The results were expressed as percentage of the control set at 100%.

The cytotoxicity of phytochemical compounds towards the NCI cell line panel was evaluated by determining the IC_{50} (concentration resulting in 50% inhibition) using a modification of the sulforhodamine B assay (SRB, Sigma, MI, USA; Monks et al. 1991).

Statistical analyses

The mRNA microarray hybridization of the NCI cell line panel has been described (Scherf et al. 2000; Amundson et al. 2008) and the date das been depositied at the NCI website (http://dtp.nci.nih.gov). For hierarchical cluster analysis, 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, while the separation in the cluster tree increases with progressive dissimilarity. Previously, cluster models have been validated for gene expression profiling and for approaching molecular pharmacology of cancer (Efferth et al. 1997; Scherf et al. 2000). Hierarchical cluster analyses applying the WARD method were done with the WinSTAT program (Kalmia, Cambridge, MA, USA). Missing values were automatically omitted by the program, and the closeness of two joined objects was calculated by

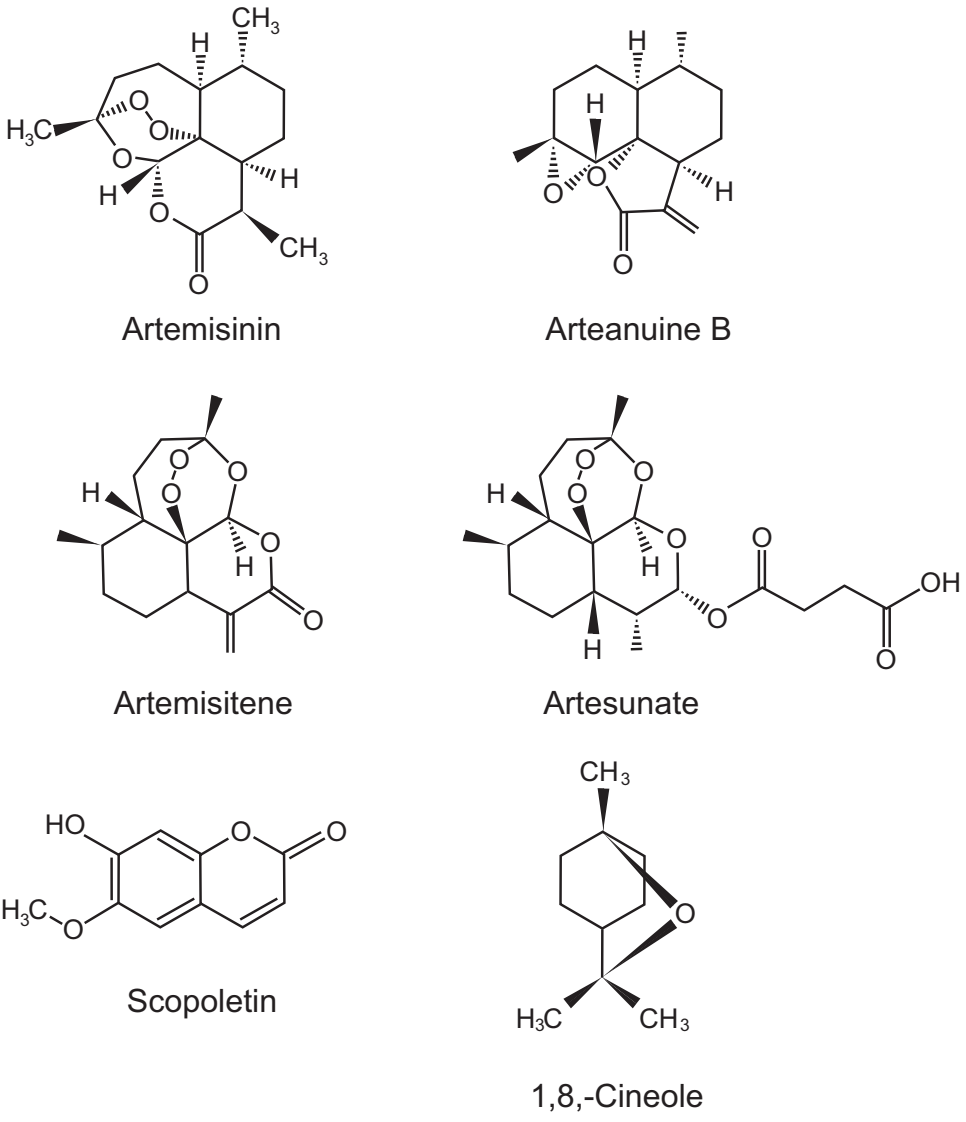

Fig. 1. Chemical structures of artemisinin, arteanuine B, artemisitene, 1,8-cineole, and scopoletin.

the number of data points they contained. In order to calculate distances between all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean = 0 and a variance = 1.

For COMPARE analysis, the mRNA expression values of genes of interest and IC_{50} values for artemisinin, arteanuine B, artemisitene, scopoletin, and 1,8-cineole of the NCI cell lines were selected from the NCI database (http://dtp.nci.nih.gov). The mRNA expression has been determined by microarray analyses as reported (Scherf et al. 2000). COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the NCI cell lines. The methodology has been described previously in detail (Wosikowski et al. 1997). Briefly, every gene of the NCI microarray database was ranked for similarity of its mRNA expression to the IC_{50} values for the corresponding compound. To derive COMPARE rankings, a scale index of correlations coefficients (*R*-values) was created. In the standard COMPARE approach, greater mRNA expression in cell lines correlate with enhanced drug resistance, whereas in reverse COMPARE analyses greater mRNA expression in cell lines indicated drug sensitivity.

Pearson's correlation test was used to calculate significance values and rank correlation coefficients as relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT Program (Kalmia).

Results

Cytotoxicity of extracts and single constituents of Artemisia annua towards trypanosomes

Dichloromethane or methanol extracts of six different *Artemisia annua* samples of different origin have been tested for their activity to inhibit the growth of trypanosomes. All extracts inhibited trypanosomal growth in a dose-dependent manner, albeit at different efficacy (Fig. 2). The IC_{50} values were calculated from the dose response curves (Table 1, Supplementary file). The dichloromethane extracts were more cytotoxic (range of IC_{50}: 1.8–14.4 μg/ml) than the methanol extracts (range of IC_{50}: 10.8–77.5 μg/ml).

Three phytochemicals have been included into the analysis, which have been described as constituents of *Artemisia annua* (Dr. Duke's Phytochemical and Ethnobotanical Databases; http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl), *i.e.* artemisinin, 1,8-cineole, and scopoletin. As shown in Fig. 3 and

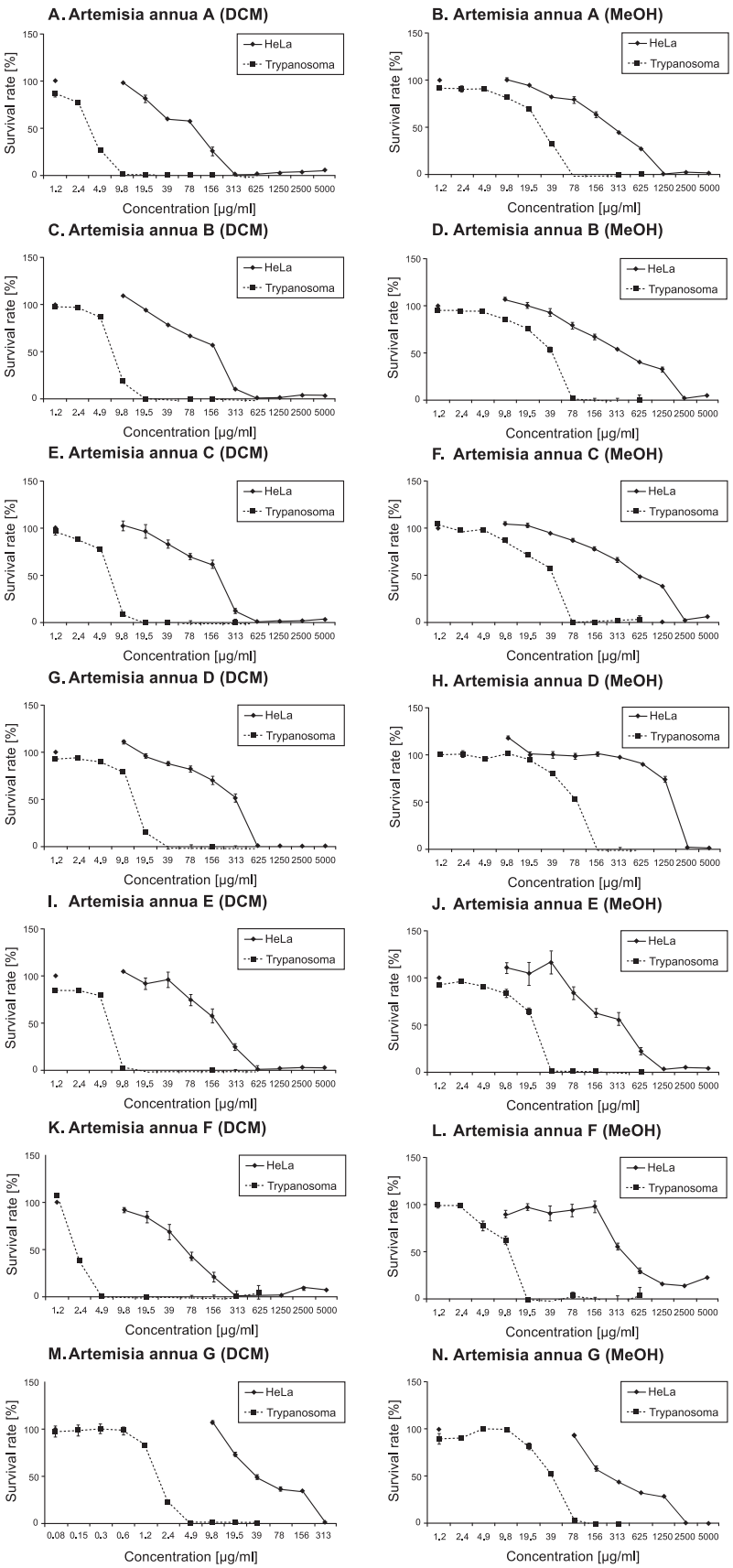

Fig. 2. Dose response curves for *Artemisia annua* extracts of human HeLa cervical carcinoma and TC221 trypanosomal cells as determined by the MTT assay. *Artemisia annua* specimen A (origin: China; purchased in Germany, harvest date: unknown); *Artemisia annua* specimen B (origin: non-governmental organization Anamed; grown in Tansania/Bunda; pulverized leaves; harvest date: 2008). *Artemisia annua* specimen C (origin: Anamed; grown in Winnenden, Germany, screen leftovers and thin caulis, harvest date: 2007). *Artemisia annua* specimen D (origin: Anamed; grown in Winnenden, Germany, chaff and thick caulis, harvest date: 2007). *Artemisia annua* specimen E (origin; China; purchased in Shanghai, China; harvest date: 2005). The plant samples were subjected to extraction by dichloromethane or methanol.

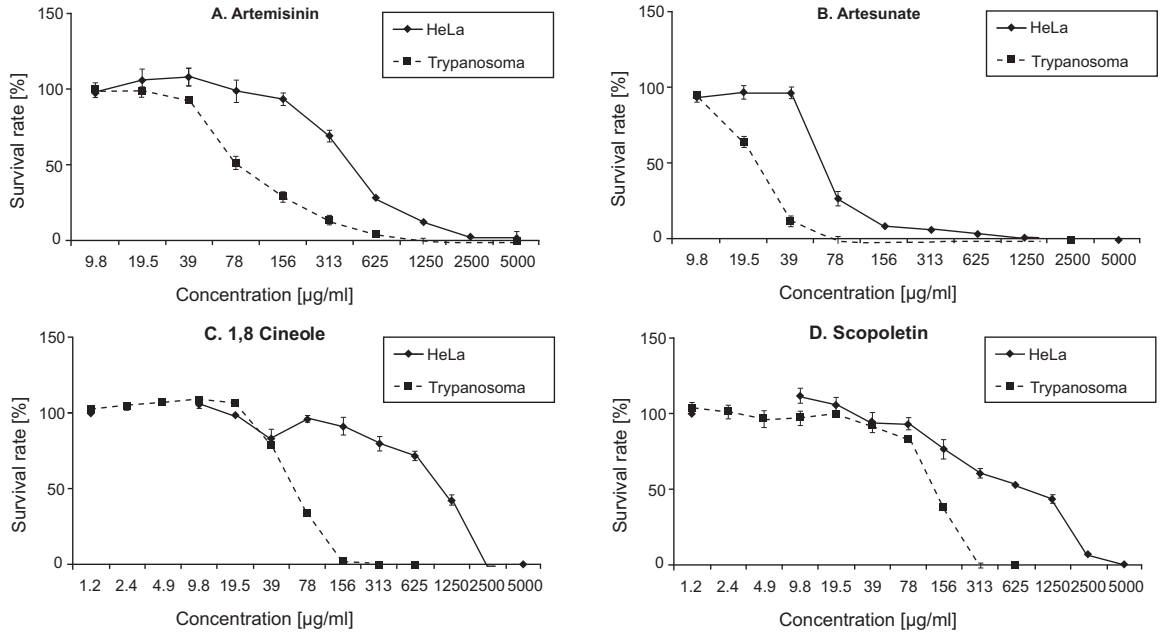

Fig. 3. Dose response curves for phytochemical constituents of *Artemisia annua* extracts of human HeLa cervical carcinoma and *Trypanosoma* cells as determined by the MTT assay.

Table 1, Supplementary file, all compounds inhibited trypanosomal growth. Of them, 1,8-cineole was the most potent one with an IC_{50} value of 64.6 μg/ml. As control drug, the semisynthetic artemisinin-derivative artesunate was used. The IC_{50} value of artesunate for trypanosomes was 2.3 μg/100 μl).

Cytotoxicity of single constituents and extracts of Artemisia annua towards HeLa cervical cancer cells

Furthermore, we tested the activity of the *Artemisia* extracts towards HeLa cells (Fig. 2). All extracts tended to be less active towards HeLa cells as compared to trypanosomes. Comparable to the results obtained for trypanosomes, dichloromethane extracts were more active towards HeLa cells than methanol extracts. The range of IC_{50} values was 54.1–275.5 μg/ml for dichloromethane extracts and 276.3–1540.8 μg/ml for methanol extracts.

The dose–response curves of individual terpenoids are shown in Fig. 3. The IC_{50} values in Table 1, Supplementary file show that artemisinin was more cytotoxic than 1,8-cineole or scopoletin. Artesunate as control drug revealed the highest cytotoxicity.

In addition to extracts of *Artemisia annua* and its chemical constituents, we also tested established trypanosomal drugs as positive control. The IC_{50} values for diminazene, DL-α-difluoromethylornithine, metronidazole, ornidazole, and suramin in TC221 cells were in a range of 0.9–18.8 μg/ml (Table 1, Supplementary file). Remarkably, the activity of dichloromethane extracts of *Artemisia annua* (IC_{50} range: 1.8–14.4 μg/ml) was comparable to that of these established drugs. The activity of these trypanosomal drugs towards HeLa cancer cells was weak (IC_{50} range: 170.6–1502.6 μg/ml).

A phytochemical investigation of the extracts by GLC–MS revealed artemisinin, arteanuine B, and scopoletin in all extracts (Table 1). Artemisitene and 1,8-cineole, both of which have been reported as components of *Artemisia annua* (http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl) were not detected in our samples.

Table 1
Relative abundance of artemisinin, arteanuine B and scopoletin in dichloromethane and methanol extracts of different *Artemisia annua* specimens as measured by GLC–MS.

Specimen	Solvent	Artemisinin	Arteanuine B	Scopoletin
A	MeOH	9.96%	9.96%	80.07%
A	CH_2Cl_2	18.75%	39.20%	42.05%
B	MeOH	47.88%	0%	52.12%
B	CH_2Cl_2	92.60%	0%	7.40%
C	MeOH	5.87%	0%	94.13%
C	CH_2Cl_2	35.41%	6.6%	57.98%
D	MeOH	6.93%	0%	93.07%
D	CH_2Cl_2	6.40%	0%	93.60%
E	MeOH	50.32%	7.62%	42.05%
E	CH_2Cl_2	59.75%	13.70%	26.55%

CH_2Cl_2, dichloromethane; MeOH, methanol.

Cytotoxicity of phytochemicals from Artemisia annua towards the NCI cell line panel

In addition to HeLa cervical carcinoma cells, we investigated the activity of artemisinin, arteanuine B, artemisitene 1,8-cineole, and scopoletin towards cell lines of different other tumor origin. The IC_{50} values for artemisinin, 1,8-cineole and scopoletin as well as two additional compounds, which are also constituents of *Artemisia annua* (arteanuine B and artemisitene) have been determined over a dose range of 10^{-8}–10^{-4} M in the NCI panel of tumor cell lines and deposited at the database of the NCI's Developmental Therapeutics Program (www.dtp.nci.nih.gov). The $\log_{10}$ IC_{50} mean values for these cell lines grouped according to their tumor type are shown in Table 2. Across all cell lines, artemisitene was the most cytotoxic compound, whereas artemisinin and scopoletin were less active. Arteanuine B and 1,8-cineole showed intermediate inhibitory activity. Interestingly, artemisitene and 1,8-cineole exhibited different activity profiles. Leukemia cell lines were most sensitive towards arteanuine B and artemisitene, whereas breast cancer cell lines were most affected by 1,8-cineole. Renal cancer cells were most resistant towards arteanuine B and artemisitene, and ovarian cancer cells showed the lowest inhibition by 1,8-cineole (Table 2).

The IC_{50} values of these cell lines were subjected to Pearson's correlation test to investigate, whether cell lines resistant to one

Table 2
50% inhibition concentration ($\log_{10}$ IC_{50}) values (M) for five phytochemicals of the NCI cell line panel grouped according to tumor types. The $\log_{10}$ IC_{50} values (mean ± SEM) were determined by the sulforhodamine assay.

	Artemisinin	Scopoletin	1,8-Cineole	Arteanuine B	Artemisitene
All tumors	−4.059 (±0.013)	−4.172 (±0.037)	−4.937 (±0.061)	−5.134 (±0.036)	−5.372 (±0.045)
Leukemia	−4.243 (±0.067)	−4.334 (±0.133)	−4.646 (±0.094)	−5.425 (±0.074)	−5.881 (±0.090)
Breast Ca	−4.053 (±0.039)	−3.928 (±0)	−6.191 (±0)	−5.164 (±0.103)	−5.564 (±0)
Colon Ca	−4.019 (±0.012)	−4.102 (±0.098)	−4.656 (±0.090)	−5,187(±0.104)	−5.451 (±0.109)
Melanoma	−4.002 (±0.002)	−4.077 (±0.046)	−4.735 (±0.146)	−5.275 (±0.084)	−5.425 (±0.080)
Brain tumors	−4.026 (±0.026)	−4.247 (±0.129)	−4.656 (±0.159)	−5.087 (±0.146)	−5.266 (±0.076)
Lung Ca	−4.078 (±0.030)	−4.209 (±0.089)	−4.950 (±0.122)	−4.904 (±0.069)	−5.227 (±0.106)
Ovarian ca	−4.060 (±0.031)	−4.096 (±0.062)	−4.370 (±0.068)	−4.958 (±0.095)	−5.137 (±0.136)
Renal Ca	−4.028 (±0.016)	−4.223 (±0.128)	−5.025 (±0.216)	−4.519 (±0.081)	−4.077 (±0.055)

Table 3
Cross-resistance profile of the NCI cell line panel towards five phytochemicals from *Artemisia annua* as determined by correlating the IC_{50} values by Pearson's correlation test.

		Artemisinin	Arteanuine B	Artemisitene	Scopoletin
1,8-Cineole	*P*-Value	−0.121	−0.152	0.232	0.229
	R-Value	0.190	0.152	0.043	0.045
Scopoletin	*P*-Value	0.070	−0.117	0.231	
	R-Value	0.305	0.215	0.042	
Artemisitene	*P*-Value	0.330	0.347		
	R-Value	0.007	0.008		
Arteanuine B	*P*-Value	0.263			
	R-Value	0.025			

phytochemical were also resistant to another compound. The rationale behind this approach was to test the cross-resistance profile of the cell lines. Although the relationships between artemisinin, arteanuine B and artemisitene revealed *P*-levels below 0.05, the correlation coefficients where rather weak ($R < 0.6$; Table 3). The IC_{50} values for 1,8-cineole and scopoletin did not correlate with those for artemisinin. Although scopoletin cytotoxicity was associated with those of 1,8-cineole and artemisitene ($P = 0.04$), the correlation coefficients were weak ($R < 0.03$).

As a control experiment, when the cross-resistance profiles of the well-known phytochemical and established anti-cancer drug, camptothecin and its derivatives (topotecan, irinotecan, and SN-38) were analyzed, significant relationships at sufficient high correlation coefficients were found ($P < 0.05$ and $R > 0.6$; Table 4), indicating pronounced cross-resistance among these drugs.

Cluster analysis of IC_{50} values of the NCI cell line panel for constitutents of Artemisia annua

To mimic the activity of several compounds in the plant by a computational approach, we subjected the IC_{50} values of arteanuine B, artemisitene, 1,8-cineole and scopoletin to hierarchical cluster analysis. The intention was to investigate whether or not clusters with high IC_{50} values for these four phytochemicals also reveal a significant probability for high IC_{50} values for artemisinin. If cell lines resistant to artemisinin were also resistant to the other compounds, these cell lines would cluster together in one of the dendrogram branches obtained. *Vice versa*, sensitive cell lines would cluster together in another branch of the dendrogram.

Table 4
Cross-resistance profile of the NCI cell line panel towards camptothecin, SN-38, irinotecan, and topotecan as control drugs.

		Camptothecin	Topotecan	Irinotecan
SN-38	*P*-Value	0.718	0.746	0.594
	R-Value	7.49×10^{-11}	5.69×10^{-12}	3.49×10^{-7}
Irinotecan	*P*-Value	0.698	0.682	
	R-Value	1.24×10^{-11}	5.38×10^{-11}	
Topotecan	*P*-Value	0.855		
	R-Value	4.1713×10^{-21}		

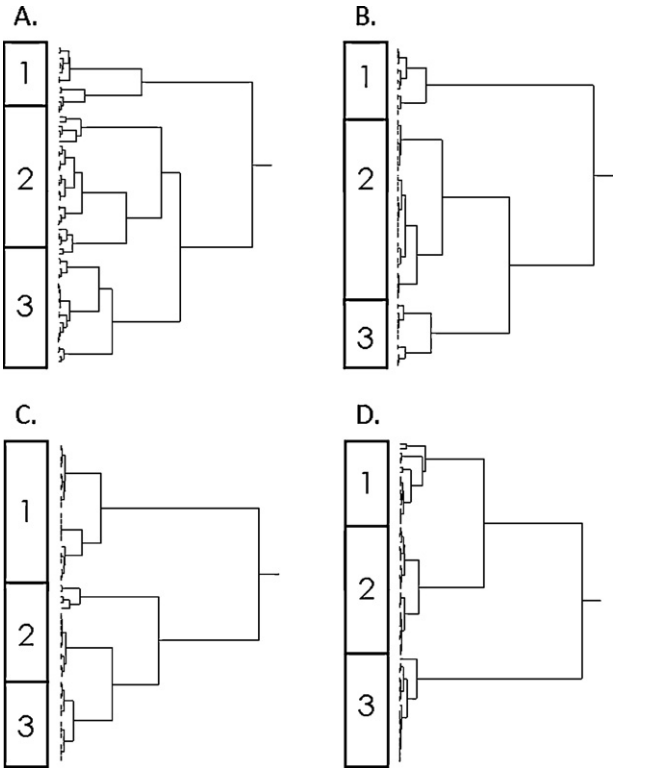

Fig. 4. Dendrograms obtained by hierarchical cluster analysis of $\log_{10}$ IC_{50} values for phytochemical constituents of *Artemisia annua* of cancer cell lines of the NCI panel. The dendrograms were obtained by clustering the IC_{50} values for (A) arteanuine B, artemisitene, 1,8-cineole, and scopoletin; (B) artemisinin-related compounds only (arteanuine B and artemisitene) and (C) artemisinin-unrelated compounds only (1,8-cineole and scopoletin). As a control experiment, the $\log_{10}$ IC_{50} values for the clinically established camptothecin derivatives, topotecan and irinotecan were clustered (D). The $\log_{10}$ IC_{50} median values of each compound were used as cut-off values to define cell lines as being sensitive or resistant.

The dendrogram in Fig. 4A shows three distinct branches. Most importantly, none of them significantly correlated with the IC_{50} values for artemisinin. To test whether these cluster branches contain cell lines with different sensitivity to arteanuine B, artemisitene, 1,8-cineole or scopoletin. The distribution of cell lines among the three dendrogram branches in Fig. 4A shows that clusters 1 and 2 contained cell lines resistant to arteanuine B, artemisitene, or scopoletin (Table 5). Cluster 3 contained cell lines sensitive to the three compounds. These relationships were statistically significant (Table 5, χ^2 test). A significant relationship to 1,8-cineole was not observed in this dendrogram. Even if we subjected only the IC_{50} values for the two artemisinin-related compounds, arteanuine B and artemisitene, to hierarchical cluster analysis, no significant rela-

Table 5
Separation of clusters of the NCI cell line panel obtained by hierarchical cluster analysis shown in Fig. 4 in comparison to phytochemical constituents of *Artemisia annua*. The IC_{50} median values of each compound were used as cut-off values to define cell lines as being sensitive or resistant. n.s., not significant ($P > 0.05$).

		Sensitive	Resistant	χ^2 test
Clustering of all four phytochemicals				
Artemisinin	Cluster 1	3	11	
($n = 63$)	Cluster 2	10	18	
	Cluster 3	12	9	n.s.
Arteanuine B	Cluster 1	5	6	
($n = 56$)	Cluster 2	5	20	
	Cluster 3	18	2	$P = 1.76 \times 10^{-5}$
Artemisitene	Cluster 1	3	11	
($n = 56$)	Cluster 2	17	10	
	Cluster 3	8	7	$P = 0.040$
1,8-Cineole	Cluster 1	3	11	
($n = 56$)	Cluster 2	16	10	
	Cluster 3	9	7	n.s.
Scopoletin	Cluster 1	0	14	
($n = 56$)	Cluster 2	16	11	
	Cluster 3	12	3	$P = 0.001$
Clustering of arteanuine B and artemisitene				
Artemisinin	Cluster 1	5	9	
($n = 63$)	Cluster 2	13	23	
	Cluster 3	7	6	n.s.
Arteanuine B	Cluster 1	2	10	
($n = 55$)	Cluster 2	13	17	
	Cluster 3	13	0	$P = 8.05 \times 10^{-5}$
Artemisitene	Cluster 1	0	14	
($n = 54$)	Cluster 2	22	8	
	Cluster 3	5	5	$P = 3.48 \times 10^{-5}$
Clustering of 1,8-cineole and scopoletin				
Artemisinin	Cluster 1	9	15	
($n = 55$)	Cluster 2	0	5	
	Cluster 3	14	12	n.s.
1,8-Cineole	Cluster 1	3	22	
($n = 56$)	Cluster 2	3	2	
	Cluster 3	22	4	$P = 1.3 \times 10^{-9}$
Scopoletin	Cluster 1	5	19	
($n = 56$)	Cluster 2	5	0	
	Cluster 3	18	9	$P = 3.09 \times 10^{-4}$
Control clustering of topotecan, irinotecan and SN-38				
Camptothecin	Cluster 1	1	17	
($n = 69$)	Cluster 2	11	17	
	Cluster 3	23	0	$P = 4.36 \times 10^{-9}$
Topotecan	Cluster 1	1	17	
($n = 69$)	Cluster 2	12	16	
	Cluster 3	23	0	$P = 6.40 \times 10^{-9}$
Irinotecan	Cluster 1	2	16	
($n = 69$)	Cluster 2	11	17	
	Cluster 3	23	0	$P = 2.38 \times 10^{-8}$
SN-38	Cluster 1	0	17	
($n = 59$)	Cluster 2	10	12	
	Cluster 3	20	0	$P = 8.46 \times 10^{-9}$

tionship to IC_{50} values for artemisinin was observed in the cluster tree (Fig. 4B), whereas the distribution of cell lines sensitive or resistant to arteanuine or artemisitene was statistically different (Table 5). Similarly, clustering of IC_{50} values for 1,8-cineole and scopoletin did not result in a dendrogram, which separates cell lines according to their resistance or sensitivity towards artemisinin (Fig. 4C). However, significant relationships were obtained for 1,8-cineole and scopoletin (Table 5). As a control, we subjected the IC_{50} values for topotecan, irinotecan and SN-38 to hierarchical cluster analysis. As expected, the dendrogram branches containing cell lines resistant to topotecan, irinotecan or SN-38 were also resistant to camptothecin with statistical significance (Fig. 4D). *Vice versa*, cell lines sensitive to topotecan, irinotecan or SN-38 were also sensitive to camptothecin.

mRNA microarray and COMPARE analyses

We further investigated the microarray-based transcriptome-wide mRNA expression by COMPARE analyses to test, whether sensitivity and resistance to the compounds were correlated with expression of similar or different sets of genes. First, standard COMPARE analyses were performed. Lowest IC_{50} values) of cell lines were correlated with the lowest mRNA expression levels of genes. Then, a reverse COMPARE analysis was done which correlated lowest IC_{50} values with the highest gene expression level. Genes with correlation coefficients of $R > 0.6$ (standard COMPARE) and $R < -0.6$ (reverse COMPARE) are listed in Table 6. Importantly, no genes appeared in association with more than one of the phytochemicals, indicating that different genes may determine cellular response to these phytochemicals and that the weak or missing cross-resistance was reflected at genetic level.

Chemoprofiling of different Artemisia species

As exemplarily shown for five phytochemicals from *Artemisia annua*, the cellular response towards these compounds was considerably different. Therefore, we attempted to establish a chemoprofile for different *Artemisia* species. We subjected the chemical compositions of 11 *Artemisia* species (Dr. Duke's Phytochemical and Ethnobotanical Databases; http://www.ars-

Table 6
Genes identified by standard or reverse COMPARE analyses whose mRNA expression in the NCI cell line panel correlated with IC_{50} values for phytochemical constituents of *Artemisia annua*.

COMPARE coefficient	ID	GenBank	Symbol	Name	Function
Artemisinin – standard COMPARE					
0.819	GC54762	AB023220	*USP20*	Ubiquitin specific peptidase 20	Ubiquitin thiolesterase, peptidase
0.771	GC183783	NM005187	*CBFA2T3*	Core-binding factor, runt domain, a subunit 2; translocated to, 3	Transcription factor
0.766	GC96878	U43185	*STAT5A*	Signal transducer and activator of transcription 5	Signal transducer and transcription factor
0.765	GC101502	Z22576	*CD69*	CD69 molecule	Transmembrane receptor
0.756	GC100401	X59834	*GLUL*	Glutamate-ammonia ligase (glutamine synthetase)	Glutamate ammonia ligase
0.753	GC182328	NM003189	*TAL1*	T-cell acute lymphocytic leukemia 1	Transcription regulator
0.752	GC166288	AL590118	*SERHL2*	Serine hydrolase-like 2	Serine hydrolase
0.742	GC90143	M6358990	None	Not specified	Unknown
0.734	GC31019	AF054186	*EEF1E1*	Eukaryotic translation elongation factor 1 epsilon 1	Translation factor
0.734	GC33623	X07109	*PRKCB1*	Protein kinase C, β 1	Calcium-activated protein kinase
0.733	GC165496	AL161952	None	Not specified	Unknown
0.727	GC182816	NM003888	*ALDH1A2*	Aldehyde dehydrogenase 1 family, member A2	Oxidoreductase
0.725	GC183873	NM005320	*HIST1H1D*	Histone cluster 1, H1d	Involved in histone condensation
0.723	GC187047	NM016520	*C9orf78*	Chromosome 9 open reading frame 78	Unknown
0.72	GC36324	Z93241	None	Not specified	Unknown
0.711	GC81738	AJ245416	*LSM2*	LSM2 homologue, U6 small nuclear RNA associated (S. cerevisiae)	Protein kinase; involved in pre-mRMA splicing
0.71	GC191046	U08626	None	Not specified	Unknown
0.706	GC186656	NM015905	*TRIM24*	Tripartite motif-containing 24	Transcription coactivator
0.7	GC32454	M6358990	None	Not specified	Unknown
0.7	GC29282	Z82206	None	Not specified	Unknown
0.693	GC156430	AI335888	*ATP9B*	ATPase, class II, type 9B	ATPase
0.686	GC188575	NM020993	*BCL7A*	B-cell CLL/lymphoma 7A	Putative F-actin cross-linking protein
0.676	GC182953	NM004117	*FKBP5*	FK506-binding protein 5	FK506 binding
0.668	GC174612	BF056790	*LOC91431*	Prematurely terminated mRNA decay factor-like	Zinc ion binding
0.668	GC99087	W60897	*ZNRD1*	Zinc ribbon domain containing 1	Transcription regulator
0.66	GC32262	AF001862	*FYB*	FYN-binding protein (FYB-120/130)	Adapter protein of FYN and LCP2 signaling
Artemisinin – reverse COMPARE					
−0.63	GC11521	H29810	None	Not specified	Unknown
−0.601	GC13148	H55766	None	Not specified	Unknown
Artemisitene – standard COMPARE					
0.712	GC31589	T89651	None	Transcribed locus, strongly similar to NP_775369.1 ribosomal protein L36A	Unknown
0.669	GC37806	D14530	*RPS23*	Ribosomal protein S23	Structural constituent of ribosome
0.644	GC30164	AF054187	*NACA*	Nascent polypeptide-associated complex a subunit	Binds nascent proteins emerging from ribosome
0.634	GC37651	Z25749	None	Not specified	Unknown
0.628	GC15116	N68924	None	Not specified	Unknown
0.628	GC148889	AA524093	*FBXO41*	F-box protein 41	Component of ubiquitin ligase complex
0.626	GC192507	Z98950	None	Not specified	Unknown
0.624	GC16228	W78173	None	Not specified	Unknown
0.62	GC34926	X79563	*RPS21*	Ribosomal protein S21	Structural constituent of ribosome
0.62	GC38852	AB019409	None	Not specified	Unknown
0.618	GC34785	AC004537	None	Not specified	Unknown
0.616	GC37574	S79522	*RPS27A*	Ribosomal protein S27a	Structural constituent of ribosome
0.612	GC30713	U68140	*NVL*	Nuclear VCP-like	Unknown
0.61	GC17766	W87741	*MYC*	Avian myelocytomastosis viral (v-myc) oncogene homologue	Transcription factor
0.608	GC37836	U59151	*DKC1*	Dyskeratosis congenita 1, dyskerin	Required for ribosome biogenesis and telomere maintenance
0.605	GC36655	U14966	*RPL5*	Ribosomal protein L5	Structural constituent of ribosome
0.604	GC36609	X80822	*RPL18A*	Ribosomal protein L18a	Structural constituent of ribosome
0.602	GC30254	AA044823	*RPL27*	Ribosomal protein L27	Structural constituent of ribosome
Artemisitene – reverse COMPARE					
−0.653	GC182056	NM002844	*PTPRK*	Protein tyrosine phosphatase, receptor type, K	Regulation of cell contact and adhesion
−0.627	GC18210	AA009800	*GSTT2B*	Glutathione S−transferase theta 2B	Detoxification of electrophiles. Phase II enzyme
−0.615	GC97127	U53204	*PLEC1*	Plectin 1, intermediate filament binding protein 500 kDa	Structural constituent of muscle
−0.609	GC91265	N26926	*GNA11*	Guanine nucleotide binding protein (G protein), a 11 (Gq class)	Signal transducer

Table 6 (*Continued*)

COMPARE coefficient	ID	GenBank	Symbol	Name	Function
−0.601	GC31852	AF037339	*CLPTM1*	Cleft lip and palate associated transmembrane protein 1	Cell differentiation
Arteanuine B – reverse COMPARE					
−0.619	GC60519		*ZNF488*	Zinc finger protein 488	Transcriptional repressor
1,8-Cineole – standard COMPARE					
0.646	GC147599	AF169689	*PCDHA6*	Protocadherin α 9	Cell adhesion
0.634	GC176788	BF792773	*FIBCD1*	Fibrinogen C domain containing 1	Receptor binding activity
0.627	GC167434	AV710838	*BCO2*	β-Carotene oxygenase 2	Oxidoreductase
0.615	GC97912	U91512	*NINJ1*	Ninjurin 1	Cell adhesion
0.612	GC165127	AL136870	*KIAA1787*	KIAA1787 protein	Unknown
0.612	GC167406	AV706915	*MTHFD2L*	Methylenetetrahydrofolate dehyhdro-genase ($NADP^+$-dependent) 2-like	Oxidoreductase
0.602	GC98312	W23068	*HSPB8*	Heat shock protein 22 kDa protein 8	Chaperone
0.601	GC160320	AI831738	*DDX59*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 59	ATP-dependent helicase
1,8-Cineole – reverse COMPARE					
−0.683	GC64100	AI375128	*FSD2*	Fibronectin type III and SPRY domain containing 2	Unknown
Scopoletin – standard COMPARE					
0.618	GC183972	NM005463	None	Not specified	Unknown
0.606	GC17951	AA001636	None	Not specified	Unknown

Information on gene functions was taken from the OMIM database, NCI, USA. (http://www.ncbi.nlm.nih.gov/Omim/) and from the GeneCard database of the Weizman Institute of Science, Rehovot, Israel. http://bioinfo.weizmann.ac.il/cards/index.html).

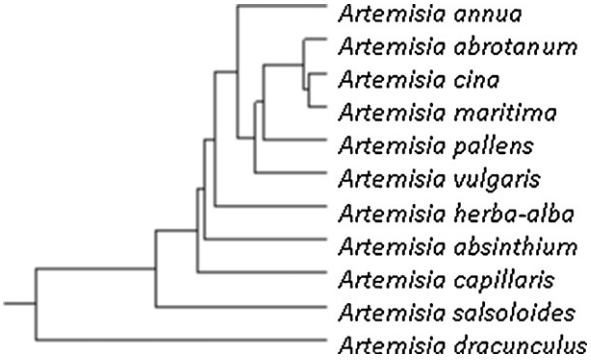

Fig. 5. Dendrogram obtained by hierarchical cluster analysis of phytochemical constituents of 11 different *Artemisia* species. Chemoprofiling of plants by cluster analysis may be termed 'herbalomics'.

grin.gov/cgi-bin/duke/farmacy2.pl) to hierarchical cluster analysis. A total number of 546 phytochemicals has been included into the analysis (Supplementary Table 2). They have been subjected to hierarchical cluster analysis. As shown in the dendrogram of Fig. 5, *Artemisia annua* clustered closely together with *A. abrotanum*, *A. cina*, *A. maritima*, *A. palens*, and *A. vulgaris* in one branch of the dendrogram, while *A. herba-alba*, *A. absinthium*, *A. capillaris*, *A. salsoloides*, and *A. dracunculus* were less closely clustered. This dendrogram demonstrates that the considerable divergence of chemical composition in the *Artemisia* species enables specific clustering and separation of the species.

Discussion

Whether or not cancer cells and protozoans develop resistance towards artemisinin-type compounds is a long standing discussion in malaria therapy. *In vitro*, it was possible to generate *Plasmodium* strains with acquired resistance towards artemisinin (Walker et al., 2000). The relevance for the *in vivo* situation was unclear, since drug-resistant *Plasmodium* strains have worldwide not been detected for many years. It was only recently that artemisinin-resistant *P. falciparum* isolates emerged at the Thailand-Cambodian border (Witkowski et al. 2010). Resistance to high dose artesunate was associated with a quiescence mechanism involving overexpression of heat shock proteins and erythrocyte surface proteins and downregulation of cell cycle regulators and DNA biosynthesis proteins. A role of genetic polymorphisms in the *pfmdr1* gene has been discussed (Pickard et al. 2003). Since drug resistance to novel and effective drugs belong to the major threats of chemotherapy, WHO recommended not to use artemisinins as monotherapy, but only in combination with other antimalarials. WHO also critically acknowledged the use of artemisinin in any other form than tablets or capsules, *i.e. Artemisia annua* tea (www.who.int/entity/medicines/publications/traditional/ArtemisiaStatement.pdf).

In this context, it is also important to address the question, whether or not artemisinin-type drugs induce resistance in cancer cells. While artemisinin cross-resistance can be tested in multidrug-resistant cell lines (Efferth et al. 2003), stable artemisinin-resistant cell line has not been described thus far. In the present investigation, we focused on phytochemicals in *Artemisia annua* in addition to artemisinin. While >50 compounds have been described in *Artemisia annua* (http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl), we exemplarily selected four compounds of interest, two artemisinin-type compounds (artemisitene and arteanuine B) and two unrelated ones (1,8-cineole, scopoletin). All of these compounds revealed cytotoxicity towards cancer and trypanosomal cells, but no cross-resistance of the NCI cell line panel was observed between artemisinin and these four phytochemicals. A general concept of drug resistance has been described by Goldie and Coldman (1985). Starting point of this seminal work were observations with bacterial strains which acquired resistance towards viruses by spontaneous mutations (Luria and Delbrück 1943). Goldie and Coldman and later on other groups developed mathematical models which explained drug resistance of tumors on the basis of spontaneous mutations of single cells. Upon drug treatment, such resistant cells have a survival advantage compared to the majority of non-mutated sensitive cells and overgrow the entire tumor cell population (Dy and Adjei 2008). Sublethal drug concentrations act as an evolutionary selection pressure for the development of resistant tumors. This can be prevented by the simultaneous treatment with a second drug. The assumption is that small subpopulations resistant to one drug are not resistant at the same time to a second drug. Therefore, they are killed by the sec-

ond drug and development of resistance to the first drug is avoided. This is the basic principle of combination chemotherapy for tumors developed in the 1970s and 1980s and still well established in clinical oncology up to now. Transferring this concept to medicinal plants *e.g. A. annua* provides a similar scenario: small subpopulations resistant to artemisinin do not survive when they are treated with 1,8-cineole. Hence, artemisinin resistance of the entire tumor cell population may be avoided. This point of view has not extensively been discussed so far in the field of phytotherapy. We have shown that artemisinin-resistant cell lines are not cross-resistant to other compounds of *Artemisia annua* such as 1,8-cineole. Therefore, when it is apparent that artemisinin-resistant subclones of a tumor can efficiently be killed by 1,8-cineole preventing the emergence of artemisinin-resistant tumors.

Our *in vitro* model with single compounds tested towards a panel of cell lines does not reflect all complex interactions in herbal mixtures of compounds. The activity of a mixture of compounds is constituted by additive and synergistic compound interactions. Synergistic interactions need a common mechanism, *e.g.* a common target where they bind to or a common pathway they inhibit. From an evolutionary point of view, synergistic compound interactions are not likely formed by chance. They need a co-evolution under appropriate selection pressure. The biosynthesis of different phytochemicals in plants with different models of action (as illustrated by different gene expression profiled) does not necessarily require co-evolutionary conditions. It can, therefore, be speculated that additive compound interactions occur with higher probability than synergistic ones.

The missing cross-resistance of the NCl cell line panel to several phytochemicals of *Artemisia annua* speaks for a sustained activity of *Artemisia annua* extracts, even if cancer cells would resist the detrimental effects of one of these compounds. Whether this result can be transferred from the *in vitro* to the *in vivo* situation remains to be seen. In addition to cellular determinants of sensitivity and resistance, the response of living organisms towards cytotoxic compounds is influenced by other factors such as immune system, angiogenesis, pharmacokinetics, etc. A clinical study with a limited number of patients showed that *Artemisia annua* tea was capable to reduce the parasitic load of malaria patients, but that the recrudescence rates were high (Mueller et al. 2004). *Artemisia annua* tea is widely used outside the official health care systems by local people and non-governmental organizations in Africa. Therefore, clinical validation is still warranted to see whether the lack of pronounced cross-resistance profiles *in vitro* among the four phytochemicals and artemisinin may indicate that herbal *Artemisia annua* extracts reveal activity towards heterogenous cancer cell populations with different genetic background and response rates towards these natural products.

This point of view is also supported by the microarray-based mRNA expression profiles identified by COMPARE and hierarchical cluster analyses. These expression profiles did not only considerably differ between artemisinin and the structurally unrelated compounds 1,8-cineole and scopoletin, but also to the related arteanuine B and artemisitene. This indicates that the molecular determinants of sensitivity and resistance of these phytochemicals are not identical and that different signaling routes and genetic networks may be active in tumor cells upon treatment with artemisinin compared to the other natural products analyzed. This is also consistent with recent pharmacogenomic data obtained for artesunate, a semisynthetic derivative of artemisinin (Sertel et al. 2010).

Among the various *Artemisia annua* extracts which have been analyzed in the present study, a considerable heterogeneity of inhibitory activity was observed. This heterogeneity was obvious among samples of different origin and points to an important aspect in phytotherapy in general. Differing bioactivities mirror biological variability between different plant individuals as well as differences in growth and cultivation conditions (soil composition, climate, harvest, processing and storage conditions). The differences observed in the present investigation clearly show the necessity for standardized cultivation conditions (*e.g.* by the rules of good agricultural practice, GAP, etc.).

In addition to heterogeneous bioactivities between different samples, we also found varying cytotoxicities of extracts from different parts of the plant obtained from the same plant sample. Most active were methanol extracts from leaves, while dichloromethane extracts from chaff and thick caulis revealed the weakest growth inhibitory activity. This is a well-known phenomenon in pharmacognosy and a specific terminology has been established characterizing the morphological structure of a plant, *e.g. flos, fructus, semen, herba, folia, summitates, ramulus, stipes, caulis, lignum, cortex, radix, bulbus, rhizoma*, and *tuber* drugs. Commonly, *Artemisia annua* is used as herbal drug (*Herba Artemisiae annuae*). The data found in our investigation showed that the leaves were more active than other parts of the plant. Hence, the leave drug (*Folia Artemisiae annuae*) is more recommendable. This is true for the cytotoxic activity towards both cancer cells and trypanosomal cells. Our data are in accord with previous results on the anti-trypanosomal activity of artemisinins and *Artemisia annua* extracts (Mishina et al. 2007; Nibret and Wink 2010). Furthermore, we showed here that phytochemicals in addition to artemisinin such as 1,8-cineole and scopoletin also revealed activity against trypanosomal cells. Hence, the bioactivity of *Artemisia annua* is not solely due to artemisinin.

Another point that has to be critically discussed is that dichloromethane extracts were more cytotoxic than the corresponding methanol extracts. This indicates that phytochemicals solved in non-polar solvents such as dichloromethane are more cytotoxic and/or present in higher amounts than compounds that can be found in polar methanol extracts. Artemisinin as best known phytochemical of *Artemisia annua* cannot be solved in polar solvents such as methanol or water. This may raise doubts about the activity of tea preparations for unsupervised self-reliant treatments. Either the tea might be inactive or other compounds than artemisinin confer bioactivity of *Artemisia annua* tea. Another possibility is that tea preparation might contain solving mediators facilitating the solubility of artemisinin.

Finally, we applied hierarchical cluster analysis for chemical profiling of diverse *Artemisia* species. Phytochemical profiling has been used in the past to test the hypothesis that the phytochemical constitution of plants can be used for taxonomy of plants, although not all species have been analyzed to the same extent. While this approach seems attractive at first sight, its taxonomic value has been controversially discussed, because the phytochemical constitution can vary within the same species due to varying external stimuli and growth conditions (Wink 2003; Wink et al. 2010). In the present investigation, we found that artemisinin was only present in *Artemisia annua*, but not in other *Artemisia* species. Although our analysis was limited to only 11 *Artemisia* species, we can conclude that artemisinin is not a lead compound for the genus *Artemisia.* These results are consistent with other investigations showing that artemisinin is present in some, but not all *Artemisia* species (Liersch et al. 1986; Luo et al. 1991; Tan et al. 1998; Erdemoglu et al. 2007; Nibret and Wink 2010). Furthermore, there was no other compound present in all *Artemisia* species analyzed, indicating that there may also be no other *Artemisia*-specific lead compound. Some natural products appeared in more than one *Artemisia* species, *e.g.* 1,8-cineole or scopoletin. These compounds have a wide distribution over many plant families.

In conclusion, the present study showed that there is no pronounced cross-resistance among different phytochemicals of *Artemisia annua*. This result obtained by cytotoxicity assays was

confirmed by microarray-based mRNA expression profiles, which revealed individual pharmacogenomic signatures with no overlap of genes for each of the phytochemicals tested.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2011.06.008.

References

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., Boyd, M.R., 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 48, 589–601.

Amundson, S.A., Do, K.T., Vinikoor, L.C., Lee, R.A., Koch-Paiz, C.A., Ahn, J., Reimers, M., Chen, Y., Scudiero, D.A., Weinstein, J.N., Trent, J.M., Bittner, M.L., Meltzer, P.S., Fornace Jr., A.J., 2008. Integrating global gene expression and radiation survival parameters across the 60 cell lines of the National Cancer Institute Anticancer Drug Screen. Cancer Res. 68, 415–424.

Baltz, T., Baltz, D., Giroud, C., Crockett, J., 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodiense* and *T. gambiense*. EMBO J. 4, 1273–1277.

Dell'Eva, R., Pfeffer, U., Vené, R., Anfosso, L., Forlani, A., Albini, A., Efferth, T., 2004. Inhibition of angiogenesis in vivo and growth of Kaposi's sarcoma xenograft tumors by the anti-malarial artesunate. Biochem. Pharmacol. 68, 2359–2366.

Dy, G.K., Adjei, A.A., 2008. Systemic cancer therapy: evolution over the last 60 years. Cancer 113 (7 Suppl.), 1857–1887.

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

Efferth, T., Sauerbrey, A., Olbrich, A., Gebhart, E., Rauch, P., Weber, H.O., Hengstler, J.G., Halatsch, M.E., Volm, M., Tew, K.D., Ross, D.D., Funk, J.O., 2003. Molecular modes of action of artesunate in tumor cell lines. Mol. Pharmacol. 64, 382–394.

Efferth, T., 2005. Mechanistic perspectives for 1,2,4-trioxanes in anti-cancer therapy. Drug Resist. Updat. 8, 85–97.

Efferth, T., 2007. Willmar Schwabe Award 2006: antiplasmodial and antitumor activity of artemisinin – from bench to bedside. Planta Med. 73, 299–309.

Efferth, T., Romero, M.R., Wolf, D.G., Stamminger, T., Marin, J.J., Marschall, M., 2008. The antiviral activities of artemisinin and artesunate. Clin. Infect. Dis. 47, 804–811.

Efferth, T., 2009. Artemisinin – a versatile weapon from traditional Chinese medicine. In: Ramawat, K.G. (Ed.), Herbal Drugs: Ethnobotany to Modern Medicine. Springer, Berlin, Heidelberg, pp. 173–193.

Erdemoglu, N., Orhan, I., Kartal, M., Adigüzel, N., Bani, B., 2007. Determination of artemisinin in selected *Artemisia* L. species of Turkey by reversed phase HPLC. Rec. Nat. Prod. 1, 2–3.

Goldie, J.H., Coldman, A.J., 1985. A model for tumor response to chemotherapy: an integration of the stem cell and somatic mutation hypotheses. Cancer Invest. 3 (6), 553–564.

Jansen, F.H., 2006. The herbal tea approach for artemisinin as a therapy for malaria? Trans. R. Soc. Trop. Med. Hyg. 100, 285–286.

Liersch, R., Soicke, H., Stehr, C., Tüllner, H.U., 1986. Formation of artemisinin in *Artemisia annua* during one vegetation period*,1. Planta Med. 52, 387–390.

Luo, S.D., Ning, B.M., Hu, W.Y., Xie, J.L., 1991. Studies on peroxides of *Artemisia lamcea*. J. Nat. Prod. 54, 573–575.

Luria, S.E., Delbrück, M., 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28 (6), 491–511.

Mishina, Y.V., Krishna, S., Haynes, R.K., Meade, J.C., 2007. Artemisinins inhibit *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* in vitro growth. Antimicrob. Agents Chemother. 51, 1852–1854.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst. 83, 757–766.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mueller, M.S., Karhagomba, I.B., Hirt, H.M., Wemakor, E., 2000. The potential of *Artemisia annua* L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. J. Ethnopharmacol. 73, 487–493.

Mueller, M.S., Runyambo, N., Wagner, I., Borrmann, S., Dietz, K., Heide, L., 2004. Randomized controlled trial of a traditional preparation of *Artemisia annua* L., (Annual Wormwood) in the treatment of malaria. Trans. R. Soc. Trop. Med. Hyg. 98, 318–321.

Nibret, E., Wink, M., 2010. Volatile components of four Ethiopian *Artemisia* species extracts and their *in vitro* antitrypanosomal and cytotoxic activities. Phytomedicine 17, 369–374.

Pickard, A.L., Wongsrichanalai, C., Purfield, A., Kamwendo, D., Emery, K., Zalewski, C., Kawamoto, F., Miller, R.S., Meshnick, S.R., 2003. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. Antimicrob. Agents Chemother. 47, 2418–2423.

de Ridder, S., van der Kooy, F., Verpoorte, R., 2008. *Artemisia annua* as a self-reliant treatment for malaria in developing countries. J. Ethnopharmacol. 120, 302–314.

RITAM Artemisia annua Task Force, Willcox, M., Falquet, J., Ferreira, J.F., Gilbert, B., Hsu, E., de Magalhães, P.M., Plaizier-Vercammen, J., Sharma, V.P., Wright, C.W., Yaode, W., 2006. Artemisia annua as a herbal tea for malaria. Afr. J. Tradit. Complement. Altern. Med. 4, 121–123.

Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T.G., Andrews, D.T., Scudiero, D.A., Eisen, M.B., Sausville, E.A., Pommier, Y., Botstein, D., Brown, P.O., Weinstein, J.N., 2000. A gene expression database for the molecular pharmacology of cancer. Nat. Genet. 24, 236–244.

Sertel, S., Eichhorn, T., Simon, C.H., Plinkert, P.K., Johnson, S.W., Efferth, T., 2010. Pharmacogenomic identification of c-Myc/Max-regulated genes associated with cytotoxicity of artesunate towards human colon, ovarian and lung cancer cell lines. Molecules 15, 2886–2910.

Tan, R.X., Zheng, W.F., Tang, H.Q., 1998. Biologically active substances from the genus *Artemisia*. Planta Med. 64, 295–302.

Ullrich, C.I., von Eitzen-Ritter, M., Jockel, A., Efferth, T., 2009. Prevention of plant crown gall tumor development by the anti-malarial artesunate of *Artemisia annua*. J. Cultivated Plants 61, 31–36.

Walker, D.J., Pitsch, J.L., Peng, M.M., Robinson, B.L., Peters, W., Bhisutthibhan, J., Meshnick, S.R., 2000. Mechanisms of artemisinin resistance in the rodent malaria pathogen *Plasmodium yoelii*. Antimicrob. Agents Chemother. 44, 344–347.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64, 3–19.

Wink, M., 2008. Evolutionary advantage and molecular modes of action of multicomponent mixtures used in phytomedicine. Curr. Drug Metab. 9, 996–1009.

Wink, M., Botschen, F., Gosmann, C., Schäfer, H., Waterman, P.G., 2010. Chemotaxonomy seen from a phylogenetic perspective and evolution of secondary metabolism. In: Wink, M. (Ed.), Biochemistry of Plant Secondary Metabolism, vol. 40, second ed. Blackwell, Annual Plant Reviews.

Witkowski, B., Lelièvre, J., Barragán, M.J., Laurent, V., Su, X.Z., Berry, A., Benoit-Vical, F., 2010. Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. Antimicrob. Agents Chemother. 54, 1872–1877.

Wosikowski, K., Schuurhuis, D., Johnson, K., Paull, K.D., Myers, T.G., Weinstein, J.N., Bates, S.E., 1997. Identification of epidermal growth factor receptor and erbB2 pathway inhibitors by correlation with gene expression patterns. J. Natl. Cancer Inst. 89, 1505–1515.