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Mechanistic perspectives for 1,2,4-trioxanes in anti-cancer therapy

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Abstract

In addition to their well-known anti-malarial activity, artemisinin and its derivatives (1,2,4-trioxanes) possess potent activity against tumor cells in the nano- to micromolar range. Candidate genes that may contribute to the sensitivity and resistance of tumor cells to artemisinins were identified by pharmacogenomic and molecular pharmacological approaches. Target validation was performed using cell lines transfected with candidate genes or corresponding knockout cells. These genes are from classes with different biological function; for example, regulation of proliferation (*BUB3*, cyclins, *CDC25A*), angiogenesis (vascular endothelial growth factor and its receptor, matrix metalloproteinase-9, angiostatin, thrombospondin-1) or apoptosis (*BCL-2*, *BAX*). Artesunate triggers apoptosis both by p53-dependent and -independent pathways. Anti-oxidant stress genes (thioredoxin, catalase, γ-glutamyl-cysteine synthetase, glutathione S-transferases) as well as the epidermal growth factor receptor confer resistance to artesunate. Cell lines over-expressing genes that confer resistance to established anti-tumor drugs (*MDR1*, *MRP1*, *BCRP*, dihydrofolate reductase, ribonucleotide reductase) were not cross-resistant to artesunate, indicating that this drug has a different target and is not subject to multidrug resistance. The *Plasmodium* translationally controlled tumor protein (*TCTP*) represents a known target protein of artemisinin and its derivatives in the malaria parasite. The microarray-based mRNA expression of human *TCTP* correlated with sensitivity to artesunate in tumor cells, suggesting that human *TCTP* contributes to response of tumor cells to the drug. The multi-factorial nature of cellular response to artemisinin and its derivatives may be beneficial to treat otherwise drug-resistant tumors and may explain why resistance development has not been observed in either cancer or malaria.

Keywords: Angiogenesis; Apoptosis; Artemisinin; Multidrug resistance; Oxidative stress; Pharmacogenomics; Sesquiterpene lactones; Traditional Chinese medicine

1. Introduction

Cancer chemotherapy is limited by the development of drug resistance in tumors and adverse side effects in patients. The search for novel anti-tumor agents that circumvent these limitations has turned to natural sources, in particular plants used in traditional folk medicines. This approach has already proven successful. Camptothecin from *Camptotheca acuminata* and paclitaxel from *Taxus brevifolia* are outstanding examples of natural products as chemotherapeutic agents (Wall and Wani, 1995).

The genus *Artemisia* L. belongs to the family of Compositae. More than 350 *Artemisia* species are known, many of which have been used in traditional folk medicines for various applications (cough, blood circulation, diuresis, hypertension, allergy, parasites, etc.). Sesquiterpene lactones, flavonoids, coumarins, acetylenes, and sterols have been isolated from *Artemisia* species, some of which reveal anti-malarial, anti-viral, anti-tumor, anti-pyretic, anti-coagulant, anti-spasmodic, and other effects (Tan et al., 1998).

Artemisinin, a sesquiterpene lactone from *Artemisia annua* L. (*qinghao*, Sweet wormwood) has raised considerable attention in past years. The plant has been used in China for more than two millenia. Its first description dates back to the third century BC. Ge Hong (281–340 AD) recommended tea-brewed leaves to treat fever and chills in his "Handbook of Prescriptions for Emergency Treatment". The "Compendium of *Materia Medica*" published by Li Shizen in 1596 cited Ge Hong's prescription. The fact that *qinghao* tea has withstood the centuries may be taken as a clue for the usefulness and

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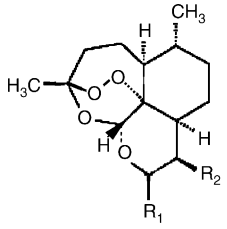

Artemisinin:	R1: =O R2: -CH_3
Artemether:	R1: -O-CH_3 R2: -CH_3
Arteether:	R1: -O-CH_2-CH_3 R2: -CH_3
Artesunate:	R1: -O-CO-CH_2-CH_2-COOH R2: -CH_3

Fig. 1. Chemical structures of 1,2,4-trioxanes.

activity of this prescription of traditional Chinese medicine (TCM).

A program for the discovery of new anti-malarial drugs from TCM launched by the Chinese government led in 1972 to the identification of artemisinin (*qinghaosu*), the active principle of *A. annua* L. (Klayman, 1985; Butler and Wu, 1992). Today, several tons per year of artemisinin are extracted from *A. annua* L. plants for pharmaceutical utilization in Asian countries (Haynes, 2001), which points to a preservation issue of wild-growing plants and which necessitates the cultivation in plantations for large scale production. Due to the low solubility of artemisinin in oil and water, several semi-synthetic derivatives have been developed, including artemether, arteether, artesunate, and others (Fig. 1). The attractiveness of the artemisinin class of anti-malarials is due to their activity against multidrug-resistant *Plasmodium falciparum* and *Plasmodium vivax* strains (Price et al., 1998). Another salient feature is the lack of severe side effects in malaria patients (Ribeiro and Olliaro, 1998), although neurotoxicity occurs in animals after prolonged treatment with supra-therapeutic doses (Brewer et al., 1994; Kamchonwongpaisan et al., 1997; Gordi and Lepist, 2004).

2. Anti-proliferative activity

During the past years, a number of tumor cell lines have been tested for their sensitivity to artemisinin and first generation derivatives (artesunate, artemether, arteether). While artemisinin and its derivatives kill malaria parasites at nanomolar concentrations, they exhibit cytotoxicity towards mammalian cells in the nanomolar to micromolar range (Sun et al., 1992; Woerdenbag et al., 1993; Zheng, 1994; Lai and Singh, 1995; Efferth et al., 1996; Beekman et al., 1997a).

Using a panel of 55 cell lines of the Developmental Therapeutics Program of the National Cancer Institute (NCI), USA, artesunate was most active against leukemia and colon cancer cell lines (mean 50% inhibition concentration (IC_{50}) values: 1 and 2 μM, respectively). Non-small cell lung cancer cell lines showed the highest mean IC_{50} value (26 μM) indicating the lowest sensitivity to artesunate in this test panel. Intermediate IC_{50} values were obtained for melanomas, breast, ovarian, prostate, CNS, and renal cancer cell lines (Efferth et al., 2001). Artesunate also inhibited the growth of medullary thyroid carcinoma cells (Rinner et al., 2004). The IC_{50} values for artesunate correlated significantly with the cell doubling times and the portion of cells in the G0/G1 or S cell cycle phases of the NCI cell lines (Efferth et al., 2003a).

Among a panel of isogenic *Saccaromyces cerevisiae* strains with defined genetic mutations in DNA repair, DNA checkpoint, and cell proliferation genes, a yeast strain with a defective mitosis-regulating *BUB3* gene showed increased artesunate sensitivity. Another strain with a defective proliferation-regulating *CLN2* gene showed increased artesunate resistance over the wild-type strain. None of the other DNA repair or DNA check-point deficient isogenic strains were different from the wild-type (Efferth et al., 2001). The conditional expression of the *CDC25A* gene by a tetracycline repressor expression vector (tet-off system) increased the sensitivity to artesunate (Efferth et al., 2003a). CDC25A is a key regulator of the cell cycle, which drives cells from the G1 into the S phase. Artesunate down-regulates the expression of CDC25A protein (Efferth et al., 2003a). These results point to the role of proliferation and the cell cycle for the cytotoxic effects of artesunate.

Several artemisinin derivatives displayed higher cytotoxicity to murine bone marrow cells than to murine Ehrlich Ascites tumor cells in a clonogenic assay (Beekman et al., 1998). The IC_{50} values for cervical cancer HeLa, uterus chorion cancer JAR, embryo transversal cancer RD and ovarian cancer HO-8910 cell lines after 48-h treatment with artemisinin and dihydroartemisinin ranged from 15 to 50 μM and from 8 to 33 μM, respectively (Chen et al., 2003).

Artemisinin potentiated 1α,25-dihydroxyvitamin-D_3-induced HL-60 leukemia cell differentiation predominantly into monocytes and all-*trans* RA-induced cell differentiation into granulocytes, respectively (Kim et al., 2003). Signal transducers involved in the differentiation process, such as extracellular-signal regulated kinase (ERK) and protein kinase C β1 (PKCB1) were affected by artemisinin.

In addition to artemisinin and first generation derivatives (artesunate, artemether, arteether, artelinate), novel derivatives have been synthesized to improve the anti-malarial activity such as deoxoartemisinin, artemisinin dimers, trimers, tetramers, cyanoarylmethyl-artemisinin derivatives and others (Lee et al., 2000; Ekthawatchai et al., 2001; Wu et al., 2001). To preserve the natural resources of *A. annua* plants, artemisinin-like endoperoxides, such as arteflene, have been

synthesized chemically (Hofheinz et al., 1994). Some of the second-generation compounds reveal remarkable cytotoxicity towards tumor cells (Beekman et al., 1997b; Posner et al., 1999, 2002, 2004; Lee et al., 2000; Galal et al., 2002; Li et al., 2003; Jeyadevan et al., 2004).

Ether-linked dimers of dihydroartemisinin caused accumulation of tumor cells in the G1 phase of the cell cycle (Beekman et al., 1997b). Growth-inhibitory artemisinin derivatives containing cyano and aryl groups also caused accumulation of P388 and A549 cells in the G1 phase (Li et al., 2001). Finally, deoxoartemisinyl cyanoarylmethyl derivatives with cytotoxic activity induced a significant accumulation of L1210 and P388 cells in the G1 phase (Wu et al., 2001).

3. Multidrug resistance

A salient feature of artemisinin and its derivatives is the lack of cross-resistance with other anti-malarials. Drug resistance is a huge problem in malaria treatment worldwide. Artemisinin and its derivatives are valuable for the treatment of otherwise unresponsive, multidrug-resistant malaria parasites (Price et al., 1998). Although *Plasmodium* strains resistant to artemisinin and its derivatives have been selected in vitro (Walker et al., 2000), resistance to this drug class has not been observed in malaria patients yet (Krishna et al., 2004).

Multidrug resistance is also an important issue in cancer chemotherapy, which tremendously hampers or even prevents the cure of many cancer patients from their disease. Therefore, the question arises, whether artemisinins are also useful to combat multidrug resistance in tumors.

Artesunate was similarly active against drug-sensitive and multidrug-resistant cell lines (Efferth et al., 2001, 2002a, 2003a) which over-express *MDR1*/P-glycoprotein (CEM/ADR5000; CEM/VCR1000, CEM/VBL_{100}), *MRP1* (CEM/E1000, HL60/AR), or *BCRP* (MDA-MB-231-BCRP). Furthermore, methotrexate-resistant CEM/MTX1500LV cells with an amplification of the dihydrofolate reductase (*DHFR*) gene and hydroxyurea-resistant CEM/HUR90 cells with over-expression of ribonucleotide reductase were not cross-resistant to artesunate. Sadava et al. (2002) observed that the cytotoxicity of artemisinin for multidrug-resistant small cell lung cancer H69VP cells was 10-fold lower than for sensitive parental H69 cells. The cross-resistance of H69VP cells to artemisinin was accompanied by a twofold increased expression of the transferrin receptor in the resistant cells compared to sensitive parental cells. Pretreatment with transferrin sensitized H69VP cells to artemisinin to near drug-sensitive levels, but no effect was seen in H69 cells.

Artemisinin, artesunate, and dihydroartemisinin increased cytotoxicity of pirarubicin and doxorubicin in P-glycoprotein-overexpressing K562/adr and in MRP1-overexpressing GLC4/adr, but not in their corresponding drug-sensitive cell lines (Reungpatthanaphong and Mankhetkorn, 2002). The drugs efficiently decreased the mitochondrial membrane potential, leading to a decrease in intracellular ATP levels. These compounds did not decrease the function of P-glycoprotein, suggesting a mechanism by which the drugs did not reverse the MDR phenomenon at the P-glycoprotein level but at the mitochondrial level (Reungpatthanaphong and Mankhetkorn, 2002). Artesunate modulated the uptake of doxorubicin in MRP1-overexpressing CEM/E1000 but not P-glycoprotein-overexpressing CEM/VCR1000 cells (Efferth et al., 2002a). Wartenberg et al. (2003) reported that artemisinin increased the tissue permeability for standard cytostatic drugs, i.e., doxorubicin, in mouse embryonic stem cell-derived embryoid bodies.

4. Pharmacogenomics

Because the molecular mechanisms of action of artemisinin and its derivatives in tumor cells are largely unknown, we applied pharmacogenomic approaches to explore the molecular determinants of sensitivity and resistance to this drug class. We mined the genome-wide mRNA expression database and correlated the expression data with the IC_{50} values for artesunate as reported by us (Efferth et al., 2001) and other artemisinin derivatives deposited in the database. This is a hypothesis-generating bioinformatical approach, which allows the identification of testable molecular targets. In general, there are two ways to reach this goal: (1) a gene hunting approach and (2) a candidate gene approach.

The assignment of anti-tumor drugs to certain pharmacological classes can be perceived as a taxonomic problem. The taxonomic classification of objects according to the similarity of features can be achieved by bioinformatic tools such as hierarchical cluster analysis and COMPARE analysis. The closeness of between-individual distances can be calculated by cluster analyses, and all objects are assembled into a cluster tree (dendrogram). By COMPARE analyses, the cytotoxic profiles of standard drugs with well-characterized modes of action are compared with those of investigational drugs with unknown modes of action (Paull et al., 1992). Profiles of drugs and cytotoxic compounds of high similarity cluster together in the dendrogram, while those with low similarity are separated in the cluster tree. This approach, developed by the NCI, has been successfully used to unravel the mode of action of novel compounds (Paull et al., 1992; Leteurtre et al., 1994; Shi et al., 1998). It can also be applied to identify small-molecule inhibitors of cancer-related proteins (Wosikowski et al., 1997; Naasani et al., 1999). Cluster and COMPARE analyses are also useful for comparing gene expression profiles with IC_{50} values for investigational drugs to identify candidate genes for drug resistance (Efferth et al., 2003b; Efferth, in press; Efferth and Kaina, 2004) and to identify prognostic expression profiles in clinical oncology (Efferth et al., 1997; Volm et al., 2002a, 2002b).

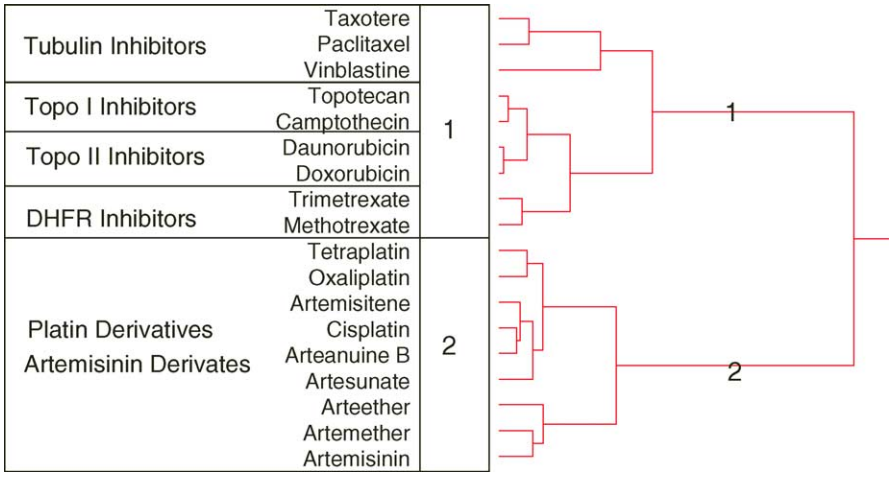

Fig. 2. Hierarchical cluster analysis of IC_{50} values of standard anti-cancer agents and 1,2,4-trioxanes of the NCI cell line panel.

A first series of experiments focused on the identification of genes, which had not previously been associated with cellular response to artemisinin or its derivatives (gene hunting approach). In an analysis of artemisinin derivatives and standard anti-tumor drugs, we showed that artemisinin and its derivatives clustered in a different area than most other standard drugs, such as DNA topoisomerase I and II inhibitors, tubulin inhibitors, and dihydrofolate reductase (DHFR) inhibitors (Fig. 2). They do, however, cluster together with platinum compounds, indicating that both drug classes may share some mechanistic similarities. While artemisinin does not cause damage of *Plasmodium* or human DNA (Yang et al., 1994; unpublished data), the cytotoxicity of platinum compounds is thought to be determined primarily by their DNA adducts. However, platinum derivatives also bind to proteins, and platinum–protein adducts contribute to cytotoxicity (Ivanov et al., 1998; Peleg-Shulman et al., 2002). Interestingly, the binding of oxaliplatin, carboplatin, and cisplatin to hemoglobin was accompanied by the release of a heme group from hemoglobin (Mandal et al., 2004). As artemisinin and its derivatives alkylate heme (Zhang et al., 1992), the clustering of both drug classes might be explained by this mechanistic similarity.

Next, we performed COMPARE analyses of the IC_{50} values for artesunate and the genome-wide mRNA expression in the NCI cell line panel. We first performed a standard COMPARE analysis in which cell lines that were most inhibited by artesunate (lowest IC_{50} values) were correlated with the highest mRNA expression levels of genes. These genes may be considered as possible candidate genes, which determine cellular sensitivity to artesunate. Afterwards, a reverse COMPARE analysis was performed: the most inhibited cell lines were correlated with the lowest gene expression levels, indicating possible drug resistance genes. Each 25 genes from standard and reverse COMPARE analyses with the highest COMPARE coefficients were subjected to hierarchical cluster analysis. The dendrogram obtained by this procedure can be divided into three major branches (Fig. 3). Then, the median $\log_{10}$ IC_{50} values for artesunate, which were not initially included in the cluster analysis, were used as a cut-off threshold to define cell lines as being sensitive or resistant. As can be seen in Table 1, the distribution of cell lines sensitive or resistant to artesunate was significantly different between the branches of the dendrograms. This is also true for artemether and arteether, whose sensitivity or resistance could be significantly predicted by this dendrogram. The distribution

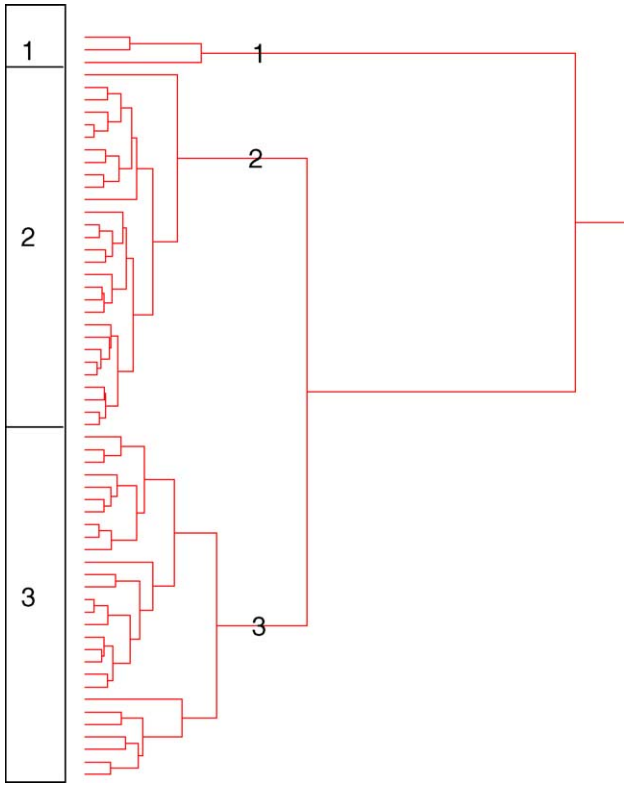

Fig. 3. Hierarchical cluster analysis of microarray-based mRNA expression of 50 genes obtained by standard and reverse COMPARE analyses. The dendrogram shows the clustering of the NCI cell line panel and indicates the degrees of relatedness between cell lines.

Table 1
Separation of clusters of the NCI cell line panel obtained by hierarchical cluster analysis shown in Fig. 3 in comparison to sensitivity to four 1,2,4-trioxanes

	Cluster 1	Cluster 2	Cluster 3	χ^2-test
Artesunate				
Sensitive	3	7	18	
Resistant	0	19	8	$p = 0.00205$
Artemether				
Sensitive	3	3	9	
Resistant	0	24	17	$p = 0.00203$
Arteether				
Sensitive	3	8	19	
Resistant	0	21	8	$p = 0.00129$
Artemisinin				
Sensitive	3	8	11	
Resistant	0	19	15	$p = 0.05526$*

The median IC_{50} values were used as cut-off thresholds to separate tumor cell lines as being "sensitive" or "resistant".
* Borderline significance ($0.05 < p < 0.1$).

of cell lines among the dendrogram predicted resistance to artemisinin with borderline significance ($p = 0.055$; Table 1). This indicates (1) that cellular response to artesunate and other artemisinin derivatives is predictable by these genes and (2) that resistance to artesunate is multi-factorial in nature.

Interestingly, one of these genes was the translationally controlled tumor protein (*TCTP*), whose *Plasmodium* homologue is a target of protein alkylation by artemisinin. The microarray-based mRNA expression of this gene correlated inversely with the IC_{50} values for artesunate in the NCI cell line panel. Tumor cell lines with high *TCTP* expression were sensitive to artesunate, while a low *TCTP* expression was associated with resistance to artesunate (Fig. 4a). *TCTP* represents a proliferation-related Ca^{2+}-binding protein, which associates transiently with microtubules during the cell cycle (Gachet et al., 1999). It has also been implicated in malignant transformation and apoptosis (Bommer and Thiele, 2004).

A third COMPARE analysis was performed using the Molecular Target Database of the NCI which contains gene expression values obtained by conventional techniques such as RT-PCR, Northern-blotting, RNAse protection assay, Western blotting, immunocytochemistry, and two-dimensional polyacrylamide gel electrophoreses (2D-PAGE). Again, a set of genes was identified by standard and reverse COMPARE analyses, which predicted sensitivity and resistance to artesunate. One of these genes was the epidermal growth factor receptor (*EGFR*). Tumor cell lines with high *EGFR* expression were significantly more resistant to artesunate than cell lines with low *EGFR* expression, indicating that *EGFR* may confer resistance to this drug (Fig. 4b). This was validated using glioblastoma cells transfected with a deletion-activated *EGFR* cDNA. The *EGFR*-transfectants were indeed more resistant to artesunate as the mock-vector transfected control cells (Efferth et al., 2003a). In addition to a role in resistance to artesunate and other drugs (Nagane

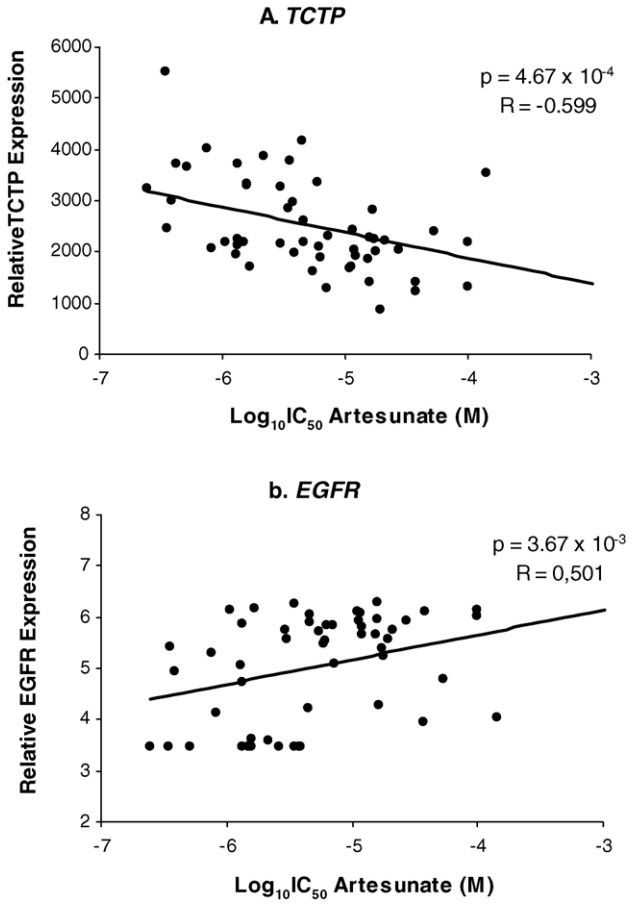

Fig. 4. Linear regression of $\log_{10} IC_{50}$ values for artesunate and mRNA expression of (a) translationally controlled tumor protein (*TCTP*) and (b) epidermal growth factor receptor (*EGFR*) in the NCI cell line panel. Significance level and correlation coefficient were calculated using Kendall's τ-test.

et al., 1996; Efferth et al., 2003a, 2003c), the activation of *EGFR*-coupled signaling routes drive mitogenic and other cancer-promoting processes, e.g., proliferation, angiogenesis, and inhibition of apoptosis (Baselga, 2002).

As the response to artesunate was affected by *EGFR*, we investigated, whether a combination of EGFR inhibitors and artesunate improves tumor cell killing. We found that the combination of artesunate with the small molecule EGFR tyrosine kinase inhibitor, OSI-774 led to supra-additive effects in glioblastoma cells transfected with a deletion-activated *EGFR* cDNA and additive effects in wild-type *EGFR* transfectants (Efferth et al., 2004a). A genomic profile of gains and losses of genomic material determined by comparative genomic hybridization was identified in nine non-transfected glioblastoma cell lines that correlated significantly with the IC_{50} values for the combination treatment of artesunate and OSI-774. Genes located at these genomic loci may serve as candidate genes who determine sensitivity and resistance to artesunate and OSI-774.

The candidate gene approach takes advantage of previously acquired knowledge on the mode of action of a drug. Then, genes of a given pathway known or assumed to be involved in a drug's action can be subjected to pharmacogenomic investigation. Concerning artesunate we have focused our efforts in three areas:

1. Genes known to affect sensitivity and resistance to established cytostatic drugs, which may also be relevant to cellular response to artemisinin and its derivatives. Therefore, we investigated 465 genes conferring multidrug resistance and other types of drug resistance, apoptosis-regulating genes, proliferation-regulating genes, oncogenes and tumor suppressor genes, DNA damage and repair genes, and cytokines and cytokine-related genes (Efferth et al., 2002b, 2003a).
2. Mechanisms operative in malaria therapy, which might also be relevant to the cytotoxic activity of artemisinin and its derivatives towards cancer cells. Since oxidative stress and heme-iron(II) play a role in killing the *Plasmodium* parasites, we analyzed whether anti-oxidant genes and genes of iron metabolism might also affect tumor cell response to artemisinin and its derivatives (Efferth et al., 2003d, 2004b; Efferth, in press; Efferth and Oesch, 2004).
3. Angiogenesis, the target of many new anti-cancer agents. It was intriguing to observe that artesunate also acts in an anti-angiogenic manner (Dell'Eva et al., 2004). We subsequently examined whether angiogenesis-related genes could predict sensitivity and resistance of tumor cells to artesunate (Efferth et al., submitted for publication).

5. Oxidative stress and iron

In malaria parasites, artemisinin acts by a two-step mechanism. It is first activated by intraparasitic heme-iron, which catalyzes the cleavage of the endoperoxide. The *Plasmodium* trophozoites and schizonts live within red blood cells. Hemoglobin serves as an amino acid source, being taken up by the parasites into food vacuoles where enzymatic degradation takes place (Semenov et al., 1998; Shenai et al., 2000). The release of heme-iron during hemoglobin digestion facilitates the cleavage of the endoperoxide moiety by a Fe(II) Fenton reaction. Breaking up the endoperoxide bridge results in the generation of typical reactive oxygen species such as hydroxyl radicals and superoxide anions. These damage membranes of food vacuoles and lead to auto-digestion (Berman and Adams, 1997; O'Neill and Posner, 2004).

In addition, the heme-iron(II)-mediates decomposition of artemisinin that generates carbon-centered radical species (Posner and Oh, 1992; Meshnick et al., 1993; Butler et al., 1998). The cleavage of the endoperoxide bond of artemisinin and its derivatives leads to the alkylation of heme and some *Plasmodium*-specific proteins, including the *P. falciparum* translationally controlled tumor protein (TCTP), histidine-rich protein (42 kDa), and PfATP6, the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) orthologue of *P. falciparum* (Asawamahasakda et al., 1994; Bhisutthibhan and Meshnick, 2001; Eckstein-Ludwig et al., 2003). Binding of artemisinin to the parasites' DNA has not been observed (Yang et al., 1994). Recent observations indicate, however, that heme-iron(II) and oxidative stress are not the only determinants of artemisinin's anti-malarial activity (Parapini et al., 2004).

To test whether oxidative stress and anti-oxidant gene expression affects the response of tumor cells to artesunate, we compared the baseline anti-oxidant mRNA gene expression in the NCI cell line panel with the IC_{50} values for artesunate (Efferth et al., 2003d). Thioredoxin reductase expression showed a significant positive correlation to the IC_{50} values for artesunate and catalase expression was inversely correlated ($p < 0.05$). WEHI7.2 mouse thymoma cells selected for resistance to hydrogen peroxide or transfected with thioredoxin, manganese superoxide dismutase, or catalase showed resistance to artesunate compared to the parental cell line.

The microarray-based mRNA expression of dihydrodiol dehydrogenase, γ-glutamylcysteine synthase (γ-GCS), glutathione S-transferases GSTM4, GSTT2, GSTZ1, and microsomal glutathione S-transferase MGST3 showed significant correlation ($p < 0.05$) to cellular response to artesunate in the NCI cell line panel. A tendency for correlation ($0.05 < p < 0.1$) was observed for GSTA1, GSTA2, GSTP1, and MGST1. MSC-HL13 cells transfected with cDNAs for heavy and light subunits of γ-GCS were more resistant to artesunate than mock control vector-transfected MSV-PC4 cells (Efferth et al., 2003a). L-Buthionine sulfoximine, a γ-GCS inhibitor that depletes cellular glutathione pools, completely reversed artesunate resistance in MSV-HL13 cells, while a partial reversion was obtained by ethacrynic acid, an inhibitor of GST (Efferth and Volm, 2005). These data support a role of oxidative stress for the anti-tumor action of artesunate and suggest that different anti-oxidant defense mechanisms act in concert to affect the cellular response to artesunate.

The results are in accord with data of Mukanganyama et al. (2001, 2002) who showed that the activity of glutathione S-transferases was inhibited by artemisinin, and that glutathione S-transferases may contribute to the metabolism of artemisinin. The authors proposed a model in which (a) artemisinin reacts with GSH resulting in oxidized glutathione; (b) the oxidized glutathione is then converted to reduced glutathione via glutathione reductase; and (c) the latter reaction may then result in the depletion of NADPH by glutathione reductase. However, not all data in mammalian systems support a role of oxidative stress for artemisinin response. Siddiqi and Pandey (1999) did not find a change in several indicators of oxidative stress in mouse liver cells after arteether treatment.

As the iron storage of tumor cells is generally much less than of erythrocytes, but is more in tumor cells compared to normal cells (Shterman et al., 1991), the question arises as to whether iron may also play a role in the in-

hibitory action of artemisinins towards tumor cells (Payne, 2003). The growth rate of the tumor was significantly retarded by daily oral administration of ferrous sulfate followed by dihydroartemisinin. No significant tumor growth retardation effect was observed in rats treated with either dihydroartemisinin or ferrous sulfate alone. The drug treatment did not significantly affect body weight compared with untreated tumor-implanted animals and no apparent toxic effect was observed after drug treatment (Moore et al., 1995).

Iron(II) glycine sulfate (Ferrosanol®) and transferrin increased the cytotoxicity of free artesunate, artesunate microencapsulated in maltosyl-β-cyclodextrin, and artemisinin toward CCRF-CEM leukemia and U373 astrocytoma cells compared with that of artemisinins applied without iron (Efferth et al., 2004b). Growth inhibition by artesunate and ferrous iron correlated with induction of apoptosis. The effect of ferrous iron and transferrin was reversed by monoclonal antibody RVS10 against the transferrin receptor, which competes with transferrin in binding to the receptor. The IC_{50} values for eight different artemisinin derivatives in the NCI cell line panel were correlated with the microarray mRNA expression of 12 genes involved in iron uptake and metabolism to identify iron-responsive cellular factors enhancing the activity of artemisinins. This analysis pointed to mitochondrial aconitase and ceruloplasmin (ferroxidase). Interestingly, exposure of artemisinin and its derivatives produces no or only marginal cytotoxicity to normal peripheral blood mononuclear cells (PBMC). In contrast, Beekman et al. (1998) found that artemisinins are more toxic to bone marrow cells compared to tumor cells. These effects may depend on the proliferative status of the cells. Leukemia cells have a higher proliferative activity than PBMC, but bone marrow stem cells are also proliferating. Human breast cells do not or hardly respond to treatment with transferrin plus dihydroartemisinin, while breast cancer cells are strictly growth-inhibited (Singh and Lai, 2001). Similar results were found with artemisinin-tagged transferrin, which inhibits leukemia cells more than normal leukocytes (Lai et al., 2005) The growth of primary human fibroblasts is almost unaffected by ART concentrations up to 100 μM (Efferth et al., 2002c). The cytotoxic action of artmisinin and its derivatives is, however, not exclusively determined by the presence of iron(II) (Beekman et al., 1997a).

The fact that the activity of artemisinin and its derivatives is increased in the presence of ferrous iron may be useful in developing new treatments. The absorption of iron is increased in growing cells and tissues and the uptake of transferrin is related to the rate of tumor cell proliferation (Aulbert et al., 1980). Cellular iron uptake and internalization are mediated by the binding of transferring–iron complexes to the transferrin receptor (CD71), expressed on the cell surface membrane, and subsequent endocytosis. CD71 expression in normal tissues is limited, e.g., to basal epidermis, endocrine pancreas, hepatocytes, Kupfer cells, testis, and pituitary, while most other tissues CD71-negative (Gatter et al., 1983). In contrast, CD71 is expressed in much larger amounts in proliferating and malignant cells (Judd et al., 1980; Trowbridge and Omary, 1981; Sutherland et al., 1981) and it is widely distributed among clinical tumors (80, Gatter et al., 1983). We found that the CD71 expression was much higher in CCRF-CEM and U373 tumor cells (48–95%) than in peripheral mononuclear blood cells of healthy donors (<2%) (Efferth et al., 2004b). This raises the attractive possibility that tumors which express more CD71 than normal cells are preferentially affected by combination treatments of transferrin or Ferrosanol® plus artemisinin derivatives. The finding that iron(II) glycine sulfate increased the action of artemisinins is interesting, since Ferrosanol® has been in clinical use for many years. Hence, artemisinins might be safely applied in combination with Ferrosanol® in a clinical setting.

6. Toxicity

Neurotoxicity has been reported in safety studies with movement disturbances and neuropathic changes occur in the hindbrain of intramuscularly treated dogs, rats, and monkeys after using extremely high doses or after prolonged exposure (Kamchonwongpaisan et al., 1997). Such effects have not been seen in malaria patients. A recent clinical safety review of 108 clinical studies enrolling 9241 patients provided ample evidence that artemisinins are safe and without serious adverse events or severe significant toxicity and especially without neurotoxicity (Ribeiro and Olliaro, 1998). Hence, it is unclear whether neurotoxicity induced by artemisinin and its derivatives in animals has clinical significance.

Nevertheless, the data obtained from animal experiments, gave reason for in vitro studies on the neurotoxic potential of artemisinins. Artemether, arteether, artemisinin, and dihydroartemisinin inhibited cell proliferation and produced a dose-related decrease in the number of neurites/extensions formed by differentiating Nb2a neuroblastoma cells (Fishwick et al., 1995). The hemin-induced increase in toxicity of dihydroartemisinin to differentiating neuroblastoma cells is accompanied by an increase in dihydroartemisinin binding to cell proteins (Fishwick et al., 1998a). Dihydroartemisinin damaged NB2a cell mitochondrial cristae and endoplasmic reticulum (Fishwick et al., 1998b; McLean and Ward, 1998), and it also depleted the filopodia-like processes projecting from the surface of the cell body and neurites (Fishwick et al., 1998b). Bhattacharjee and Karle (1999) suggested that higher intrinsic lipophilicity of artemisinin derivatives is associated with greater neurotoxicity.

Neurodegeneration appears to be induced by effects on the cytoskeleton and by reduction of intracellular ATP levels as a consequence of by mitochondrial or metabolic defects. Artemisinin additionally induces oxidative stress. The levels of reactive oxygen species and of lipid peroxidation increased in both artemisinin-sensitive neuronal brain stem cell cultures and in non-sensitive cultures (cortical neurons, astrocytes).

In astrocytes, manganese superoxide dismutase (MnSOD) and catalase expression was increased upon artemisinin challenge. In brain stem neurons, MnSOD expression was dose dependently decreased (Schmuck et al., 2002).

Both dihydroartemisinin and a combination of artemether plus hemin significantly inhibited neurite outgrowth from differentiating NB2a cells (Smith et al., 1998, 2001). The inhibition by artemether/hemin or dihydroartemisinin was prevented by the several anti-oxidants, including glutathione, L-cysteine, or *N*-acetyl-L-cysteine. L-Buthionine sulfoximine significantly increased the neurotoxic effect of non-toxic concentrations of artemether/hemin and dihydroartemisinin, suggesting that endogenous glutathione participates in the prevention of the neurotoxicity. Artemether/haemin completely depleted intracellular glutathione levels (Smith et al., 1998, 2001).

Efferth and Volm (2005) analyzed the expression of glutathione S-transferase in normal rat organs. Positive immunostaining was found in all organs analyzed, albeit with varying staining intensities and in different histological structures of the organs. GST expression in normal organs may, therefore, contribute to the good tolerability to artemisinin and its derivatives in normal organs as previously reported in a large meta-analysis with 9241 malaria patients (Ribeiro and Olliaro, 1998). Glutathione S-transferase expression in brain capillaries represents a constituent of the blood brain barrier (Carder et al., 1990) and may protect brain tissue from the detrimental effects of artemisinins. Experiments with laboratory animals, however, show that very high doses and prolonged exposure to artemisinins provoke neurotoxicity (Gordi and Lepist, 2004). At high concentrations the detoxification capability of GST might be overloaded and neurotoxic symptoms appear. This view is supported by experiments with L-buthionine sulfoximine, which significantly increases the neurotoxic effect of non-toxic concentrations of artemether and dihydroartemisinin. Smith et al. (2001) suggested that endogenous glutathione participates in the prevention of the neurotoxicity of artemisinins. It still remains open, whether treatment of cancer patients with artemisinins is also free of severe side effects as found for malaria treatment.

Established cancer drugs such as anthracyclines also form reactive oxygen species and free radicals that are responsible for the cardiotoxicity of anthracyclines. In contrast, artemisinins do not reveal cardiotoxicity. Therefore, Efferth and Oesch (2004) have compared the cytotoxic activities of different artemisinins (artemisinin, artesunate, arteether, artemether, artemisitene, and two dihydroartemisinylester stereoisomers) in 60 NCI cell lines with those of anthracyclines (doxorubicin, daunorubicin, 4′-epirubicin, idarubicin, deoxydoxorubicin, trifluoroacetyl-doxorubicin-14-valerate). The IC_{50} values of artemisinins and anthracyclines were correlated with the mRNA expression of 170 genes involved in oxygen stress response and metabolism. The genes whose expression was significantly associated to cellular drug response were subjected to hierarchical cluster analysis. One cluster contained predominantly genes with a relationship to artemisinins and another one contained genes with a relationship to anthracyclines. In a third cluster, genes correlating to both drug classes were assembled. This indicates that different sets of genes involved in oxidative stress response and metabolism may contribute to the differing toxic side effects of these drug classes.

7. Apoptosis

Since most anti-cancer drugs kill tumor cells by the induction of apoptosis, it is reasonable to assume that the same is true for artemisinin and its derivatives. There are two main pathways that trigger apoptosis (Schimmer et al., 2001; Fulda and Debatin, 2003; Green and Kroemer, 2004). Both the extrinsic and the intrinsic pathway of apoptosis are regulated by the tumor suppressor gene p53 (Haupt et al., 2003). Members of the tumor necrosis factor family activate the receptor-mediated extrinsic pathway (Shankar and Srivastava, 2004). FAS/CD95/APO-1 is the most prominent member of these death receptors. The mitochondrial intrinsic pathway is activated by stress signals or loss of survival signals. The *BCL-2* gene family is involved in the mitochondrial regulation of apoptosis. The different members of this gene family act as homo- or heterodimers either in an anti-apoptotic manner (i.e., *BCL-2*, *BCL-xL*, *BCL-w*, *A1*, *MCL-1*, *BOO*) or in a pro-apoptotic manner (i.e., *BAX*, *Bcl-xS*, *BAK*, *BOK*, *BIK*, *BAD*, *BID HRK*, *NOXA*). Mitochondrial outer membrane permeabilization results in the release of caspase-activating molecules. As a consequence, initiator caspases activate effector caspases that catalyze proteolysis ultimately leading to cell death.

Artesunate was first shown to induce apoptosis in tumor cells by Efferth et al. (1996), a result subsequently corroborated by several other research groups. Different artemisinin derivatives containing cyano- and aryl-groups induced apoptosis in murine P388 and human A549 cells (Li et al., 2001). After administration of artesunate, growth of H22 solid hepatic carcinoma in mice was inhibited, and apoptosis of liver cancer cells was increased. Immunohistochemical staining showed that the number of BCL-2-positive cells was decreased, but the number of BAX-positive cells was increased (Wang et al., 2002).

By microarray and hierarchical cluster analyses, a set of apoptosis-regulating genes was identified whose mRNA expression correlated significantly with the IC_{50} values for artesunate in the NCI cell lines (Efferth et al., 2003a). Furthermore, ART acts via p53-dependent and -independent pathways in isogenic p53+/+ p21WAF1/CIP1+/+, p53−/− p21WAF1/CIP1+/+, and p53+/+ p21WAF1/CIP1−/− colon carcinoma cells (Efferth et al., 2003a). BCL-2-transfected WEHI7.2 cells were more resistant to artesunate than mock vector-transfected control cells (Efferth et al., 2003d). This was confirmed in a subsequent study with p53 wild-type TK6 and p53 mutated WTK1 lymphoblastic cells, which revealed

similar sensitivity towards artesunate (Efferth et al., 2004b). Yamachika et al. (2004) found up-regulation of BAX, CD40 and CD40L, and p53 and down-regulation of BCL-2 upon treatment of HPV 16 immortalized and transformed human gingival epithelial (IHGK) cells with artemisinin which paralleled the induction of apoptosis.

Studies on the time and concentration dependence of artesunate induced apoptosis showed it to occur in CCRF-CEM acute-T-lymphoblastic cells after a 96 h exposure to artesunate (Efferth et al., 2004b). DNA fragmentation as measured by ELISA occurred within artemisinin- and transferrin-treated H69 lung cancer cells, with kinetics indicating apoptosis rather than necrosis (Sadava et al., 2002). The induction of apoptosis by dihydroartemisinin in MOLT-4 acute T-lymphoblastic leukemia cells was increased by transferrin as measured by a DNA diffusion assay (Singh and Lai, 2004). Necrotic death was not observed in MOLT-4 cells either. A dose-related (5–80 μM) and time-dependent (6–36 h) increase in dihydroartemisinin-induced HUVEC apoptosis was observed by flow cytometry (Chen et al., 2004a). Interestingly, artemisinin also induced death of *P. falciparum* parasites by sphingomyelinase-generated ceramide and by decreasing the parasites' glutathione level (Pankova-Kholmyansky et al., 2003).

The induction of apoptosis by artesunate was shown for human KS-IMM Kaposi sarcoma cells, whereas normal endothelial cells did not undergo apoptosis in response to artesunate treatment (Dell'Eva et al., 2004). This is in accordance with results obtained with melanoma cells and normal fibroblast cells (unpublished data).

The nuclear transcription factor κB (NF-κB) represents an inhibitor of apoptosis and confers resistance to standard anti-cancer agents (Baldwin, 2001; Pommier et al., 2004; Ravi and Bedi, 2004). As shown by band shift assays, artesunate inhibits the DNA-binding activity of the cellular NF-κB (Efferth et al., 2002c). As a result, the viral proteins IE1p72, pUL84 und pUL94 were down-regulated by artesunate. Hence, artesunate acts in an anti-viral manner by interfering with host cell signaling cascades. Other sesquiterpene lactones act as inhibitors of NF-κB (Siedle et al., 2004) and may thereby trigger apoptosis. Although not proven yet, it can be speculated that NF-κB may also play a role in artesunate-induced apoptosis.

8. Angiogenesis

In the angiogenic process, the formation of new blood vessels from pre-existing ones is essential for the supply of tumors with oxygen and nutrients and for the spread of metastatic cells throughout the body (Folkman, 1992). Angiogenesis is promoted by numerous factors including cytokines, VEGF, bFGF, PDGF, etc. and negatively regulated by angiostatin, endostatin, thrombospondin, TIMP, and others. These factors, which are produced in tumor cells as well as in surrounding stromal cells, act in a balance to promote either pro-angiogenic or anti-angiogenic processes (Relf et al., 1997). Inhibitors of angiogenesis that block angiogenic signals have been developed, and anti-angiogenic therapy strategies have raised considerable interest as valuable adjuncts to cytostatic and cytotoxic chemotherapy (Kerbel and Folkman, 2002; Broxterman et al., 2003; Shimizu and Oku, 2004).

Tumor hypoxia activates the transcription factor hypoxia-inducible factor-1α (HIF1α). This adaptation increases tumor angiogenesis to support the survival of poorly nourished cancer cells. Hypoxic tumors are resistant to radiation and many anti-cancer agents (Yu et al., 2002; Wouters et al., 2004). McCarty (2003) pointed to the fact that HIF-1α is activated during angiostatic therapy and that HIF-1α up-regulates the transferrin receptor expression. Since artemisinin is selectively toxic to iron-loaded cells, radio- and drug-resistant tumors might be selectively susceptible to attack by the iron-loading/artemisinin strategy.

Artemisinin and dihydroartemisinin significantly inhibited angiogenesis in a dose-dependent manner as demonstrated by measurement of proliferation, migration and tube formation of human umbilical vein endothelial (HUVE) cells (Chen et al., 2003). Dihydroartemisinin markedly reduced VEGF binding to its receptors on the surface of HUVEC and reduced the expression levels of two major VEGF receptors, Flt-1 and KDR/flk-1, on HUVEC. Chicken chorioallantoic membrane (CAM) neovascularization was significantly inhibited by dihydroartemisinin (Chen et al., 2004a). The inhibitory effect of artemisinin on HUVEC proliferation was stronger than that on HeLa, JAR, HO-8910 cancer cells, NIH-3T3 fibroblast cells and human endometrial cells (Chen et al., 2004b).

Wartenberg et al. (2003) investigated the anti-angiogenic effects of artemisinin in mouse embryonic stem cell-derived embryoid bodies, which are a model system for early post-implantation embryos and which efficiently differentiate into capillaries. Artemisinin dose-dependently inhibited angiogenesis in embryoid bodies and raised the level of intracellular reactive oxygen species. Furthermore, impaired organization of the extracellular matrix component laminin and altered expression patterns of matrix metalloproteinases 1, 2, and 9 were observed during the time-course of embryoid body differentiation. Artemisinin down-regulated hypoxia-inducible factor-1α and vascular endothelial growth factor (VEGF) expression, both of which control endothelial cell growth.

Anti-angiogenic effects were also shown for artesunate. It significantly inhibited CAM angiogenesis (Huan-Huan et al., 2004) and proliferation and differentiation of human microvascular dermal endothelial cells in a dose-dependent manner and reduced Flt-1 and KDR/flk-1 expression (Huan-Huan et al., 2004). Artesunate strongly reduced angiogenesis in vivo in terms of vascularization of Matrigel plugs injected subcutaneously into syngenic mice (Dell'Eva et al., 2004). Artesunate also retarded growth of human ovarian cancer HO-8910 xenografts in nude mice. Microvessel density was reduced following drug treatment with no apparent toxic-

ity to the animals. Artesunate also markedly lowered VEGF expression in tumor cells and KDR/flk-1 expression in endothelial cells as well as tumor cells (Chen et al., 2004b).

The microarray-based mRNA expression of 30 out of 89 angiogenesis-related genes correlated significantly with the cellular response to several artemisinins. Among this panel were many fundamental angiogenic regulators such as vascular endothelial growth factor C (*VEGFC*), fibroblast growth factor-2 (*FGF2*), matrix metalloproteinase 9 (*MMP9*), thrombospondin-1 (*THBS1*), hypoxia-inducing factor-α (*HIF1A*), angiogenin (*ANG*) and others. By means of hierarchical cluster analysis, expression profiles were identified that determined significantly the cellular response to artesunate, arteether, artemether, and dihydroartemisinylester stereoisomer 1. A borderline significance ($0.05 < p < 0.1$) was observed to dihydroartemisinylester stereoisomer 2 and artemisinin (Efferth et al., submitted for publication). The fact that sensitivity and resistance of tumor cells could be predicted by the mRNA expression of angiogenesis-related genes indicates that artemisinins reveal their anti-tumor effects at least in part by inhibition of tumor angiogenesis. Thioacetal artemisinin derivatives also inhibited HUVEC tube formation and exhibited anti-angiogenic effects (Oh et al., 2004).

9. Perspectives

Considerable progress has been made during the past years towards understanding the molecular modes of action of artemisinin and its derivatives against tumor cells. Diverse lines of research show that the cellular response to artemisinin and its derivatives is multi-factorial in nature (Fig. 5). This may be beneficial in treating otherwise drug-resistant tumors and may explain why the development of artemisinin resistance has not yet been encountered in cancer cell lines or malaria patients.

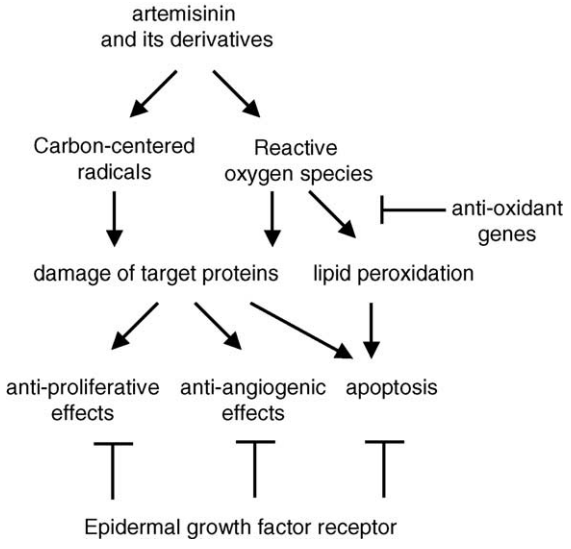

Fig. 5. Synopsis of the molecular modes of action of 1,2,4-trioxanes in tumor cells.

While most investigations have been performed with cell lines in vitro, there are few reports showing activity in vivo in xenograft tumors, e.g., Kaposi sarcoma, fibrosarcoma, or liver cancer (Moore et al., 1995; Li et al., 2001; Dell'Eva et al., 2004). These experiments were performed with high doses of the drugs. The small number of papers dealing with the in vivo anti-cancer activity of artemisinins might be a clue for weak activity in animals. At high concentrations, artemisinins seem to be active against tumors in vivo. As this drug class generally exerts low toxicity compared to established cytostatic drugs, high concentrations may not limit their use for cancer treatment. The question is, whether neurotoxicity will be a potential issue of artemisinins. The efficacy may be further enhanced by co-administration of iron(II) preparations such as Ferrosanol® or holotransferrin. Furthermore, the development of novel artemisinin derivatives with improved anti-cancer activity in vivo at tolerable side-effects is desirable.

Two case reports on the use of artesunate for the treatment of laryngeal carcinoma and uveal melanoma have been reported thus far (Singh and Verma, 2002; Berger et al., in press). Some anecdotal reports on the use of artemisinin and its derivatives in veterinary and human oncology have appeared on the internet. These reports were, unfortunately, not part of controlled clinical studies and were not published in peer-reviewed journals. More in vivo data are needed to establish the activity of artemisinin and its derivatives in living organisms, before clinical studies can be initiated.

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