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

Thomas Efferth *

German Cancer Research Center, 69120 Heidelberg, Germany

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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, angiotatin, 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.

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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 millennia. 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 Shizhen 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
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, artemenate, and others (Fig. 1). The attractiveness of the artemisinin class of anti-malarials is due to the identification of artemisinin (TCM). Artemisinin-like endoperoxides, such as arteflene, have been explored as potential antimalarial drugs.

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 (artemethane, arteether, 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; Woordenbag 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, artemisinin was most active against leukemia and colon cancer cell lines (mean 50% inhibition concentration (IC50) values: 1 and 2 μM, respectively). Non-small cell lung cancer cell lines showed the highest mean IC50 value (26 μM) indicating the lowest sensitivity to artemisinin in this test panel. Intermediate IC50 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 IC50 values for artemisinin 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 *Saccharomyces 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 artemisin sensitivity. Another strain with a defective proliferation-regulating CLN2 gene showed increased artemisin 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 tet-repressor expression vector (tet-off system) increased the sensitivity to artemisin (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 artemisin.

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 IC50 values for cervical cancer HeLa, uterus choriocarcinoma 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). Artesinin potentiated 1α,25-dihydroxyvitamin-D3-induced HL-60 leukemia cell differentiation predominantly into monocyes 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 Cβ1 (PKCβ1) were affected by artemisinin.

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

Artemisinin, artesunate, and dihydroartemisinin in- teract with ribonucleotide reductase, DHFR, and HPC (Beekman et al., 1997b; Posner et al., 1997; Naasani et al., 1999). Cluster and COMPARE analyses are also useful for comparing gene expression profiles in clinical oncology (Efferth et al., 1997; Volm et al., 2002a, 2002b).

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 IC50 values for artemisinin 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 bioinformatical 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. 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 IC50 values for investigational drugs to identify candidate genes for drug resistance (Efferth et al., 2003; 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).
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 IC₅₀ 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 IC₅₀ 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 log₁₀ IC₅₀ 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 arteether and arteether, whose sensitivity or resistance could be significantly predicted by this dendrogram. The distribution
Table 1

<table>
<thead>
<tr>
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<th>Cluster 2</th>
<th>Cluster 3</th>
<th>p²-test</th>
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<tr>
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<tr>
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<td>Resistant</td>
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<td>0.05526</td>
</tr>
</tbody>
</table>

The median IC₅₀ 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 IC₅₀ 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 Ca²⁺-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, RNAase 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 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 IC₅₀ 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.

Fig. 4. Linear regression of log₁₀ IC₅₀ 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 t-test.
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 artesinin 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 artesinin 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 artesinin and its derivatives (Efferth et al., 2003a, 2004b; Efferth, in press; Efferth and Oesch, 2004).

3. Angiogenesis, the target of many new anti-cancer agents. It was intriguing to observe that artesinin 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 artesinin (Efferth et al., submitted for publication).

5. Oxidative stress and iron

In malaria parasites, artesinin 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 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 re-
tarded by daily oral administration of ferrous sulfate fol-
lowed by dihydroartemisinin. No significant tumor growth
retardation effect was observed in rats treated with either di-
hydroartemisinin or ferrous sulfate alone. The drug treatment
did not significantly affect body weight compared with un-
treated tumor-implanted animals and no apparent toxic effect
was observed after drug treatment (Moore et al., 1995).

Iron(II) glycine sulfate (Ferrosanol®) and transferrin
corelease mediated by the binding of transferring–iron com-
plexes 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 epi-
dermis, 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 (0, 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 pos-
sibility 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 am-
piece 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 experi-
ments, gave reason for in vitro studies on the neurotoxic
potential of artemisinins. Artemether, artether, artemisinin,
and dihydroartemisinin inhibited cell proliferation and
produced a dose-related decrease in the number of neurites/extensions formed by differentiating NB2a neuroblasto-
toma 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 mitochon-
drial 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., 1999b). Bhattacharjee
and Karle (1999) suggested that higher intrinsic lipophilic-
ity 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 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, t.-cysteine, or N-acetyl-t-cysteine. t.-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/hemin completely depleted intracellular glutathione levels (Smith et al., 1998, 2001).

Efferth and Vom (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 t.-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, artemesinate, arteether, artemether, artesitene, and two dihydroartemisins) in mouse liver carcinoma cells (Efferth et al., 2003d). This indicated 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-2, 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 IC50 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+/r 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., 2003a). 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 artesinin 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 artesumin- and transferrin-treated H69 lung cancer cells, with kinetics indicating apoptosis rather than necrosis (Sadava et al., 2002). The induction of apoptosis by dihydroartesumin 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 (5–40 μM) and time-dependent (6–36 h) increase in dihydroartesumin-induced HUVEC apoptosis was observed by flow cytometry (Chen et al., 2004a). Interestingly, artesumin also induced death of P. falciparum parasites by sphingomyelinase-generated ceramide and by decreasing the parasites’ glutathione level (Pankova-Kholmysynska 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 NF-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 and 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, hFGF, PDGF, etc. and negatively regulated by angiotatin, 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 (Reif 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 artesunate is selectively toxic to iron-loaded cells, radio- and drug-resistant tumors might be selectively susceptible to attack by the iron-loading/artesunate 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 HUVECs and reduced the expression levels of two major VEGF receptors, Flt-1 and KDR/flk-1, on HUVE. Chicken choroidallantoic membrane (CAM) neovascularization was significantly inhibited by dihydroartemisinin (Chen et al., 2004a). The inhibitory effect of artesumin on HUVEC proliferation was stronger than that on HeLa, JAR, HO-8910 cancer cells, NIH-3T3 fibroblasts and human endometrial cells (Chen et al., 2004b).

Warnerberg et al. (2003) investigated the anti-angiogenic effects of artesumin in mouse embryonic stem cell-derived embryoid bodies, which are a model system for early post-implantation embryos and which efficiently differentiate into capillaries. Artesumin 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 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 Flk-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-
Stereoisomer 1. A borderline significance (0.05 < p < 0.1) was observed for dihydroartemisinylester stereoisomer 1. A borderline significance (0.05 < p < 0.1) was observed for 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 artemisinins seems 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.

References


