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Artemisinin–Second Career as Anticancer Drug?

Thomas Efferth

Johannes Gutenberg University, Institute of Pharmacy and Biochemistry, Department of Pharmaceutical Biology, Staudinger Weg 5, 55128 Mainz, Germany.

Correspondence: Thomas Efferth, Johannes Gutenberg University, Institute of Pharmacy and Biochemistry, Department of Pharmaceutical Biology, Staudinger Weg 5, 55128 Mainz, Germany. *Email*: efferth@uni-mainz.de

ABSTRACT

Artemisinin represents a showcase example not only for the activity of medicinal herbs deriving from traditional chinese medicine, but for phytotherapy in general. Its isolation from Sweet Wormwood (*qinhao*, *Artemisia annua* L.) represents the starting point for an unprecedent success story in the treatment of malaria worldwide. Beyond the therapeutic value against *Plasmodium* parasites, it turned out in recent years that the bioactivity of artemisinin is not restricted to malaria. We and others found that this sesquiterpenoid also exerts profound anticancer activity *in vitro* and *in vivo*. Artemisinin-type drugs exert multi-factorial cellular and molecular actions in cancer cells. Ferrous iron reacts with artemisinin, which leads to the formation of reactive oxygen species and ultimately to a plethora anticancer effects of artemisinins, *e.g.* expression of antioxidant response genes, cell cycle arrest (G1 as well as G2 phase arrests), DNA damage that is repaird by base excision repair, homogous recombination and non-homologous end-joining, as well as different modes of cell death (intrinsic and extrinsic apoptosis, autophagy, necrosis, necroptosis, oncosis, and ferroptosis). Furthermore, artemisinins inhibit neoangiogenesis in tumors. The signaling of major transcription factors (NF-κB, MYC/MAX, AP-1, CREBP, mTOR etc.) and signaling pathways are affected by artemisinins (*e.g.* Wnt/β-catenin pathway, AMPK pathway, metastatic pathways, nitric oxide signaling, and others). Several case reports on the compassionate use of artemisinins as well as clinical Phase I/II pilot studies indicate the clinical activity of artemisinins in veterinary and human cancer patients. Larger scale of Phase II and III clinical studies are required now to further develop artemisinin-type compounds as novel anticancer drugs.

Key words: *Artemisia annua*, Artemisinin, Cancer, Chemotherapy, *Qinhaosu*, Malaria, Phytotherapy

Abbreviations: ABCB6, ATP-binding Cassette Transporter B6; ABCG2, ATP Binding Cassette Transporter G2; AIF, Apoptosis Inducing Factor; AKT, V-Akt Murine Thymoma Viral Oncogene Homologue; AMPK, AMP-Activated Protein Kinase; Ang-1, Angiotensin 1; ARE, Arteether; ARM, Artemether; ARS, Artemisinin; ART, Artesunate; ATF4, Activating Transcription Factor 4; Bak, Bcl2 Antagonist/Killer 1; Bax, Bcl2-Associated X Protein, Pro-Apoptotic BH3-Only Bcl-2 Family Member; Bcl-2, B-cell CLL/lymphoma 2; Bcl-xL, B-cell CLL/Lymphoma-x Long; BCR/ABL, Breakpoint Cluster Region/Abl Proto-Oncogene; Bid, BH3-Interacting Domain Death Agonist; Bim, Pro-Apoptotic Bcl2-Family Member; BSO, Buthionine Sulfoximine; C/EBP β, CCAAT/Enhancer Binding Protein β; CAM, Chorioallantoic Membrane; CD, Cluster of Differentiation; CDC25B; CDK, Cyclin-Dependent Kinase; CHOP/DDIT, DNA Damage-Inducible Transcript; CIP1/WAF1, CDK-Interacting Protein 1/Wild-Type p53-Activated Fragment 1; c-JUN, Jun Proto-Oncogene; COX2, Cyclooxygenase 2; CREB, Cyclic ATP Responsive Element Binding Protein; DHA, Dihydroartesunate; DNA-PK, DNA-Dependent Protein Kinase; DR5, Death Receptor 5; E2F1, E2F Transcription Factor 1; EA, Ethacrynic Acid; EGFR, Epidermal Growth Factor Receptor; EMT, Epithelial to Mesenchymal Transition; EndoG, Endonuclease G; ERK, Extracellular Signal-Regulated Kinase; FAK, Focal Adhesion Kinase; FAS, Fas Cell Surface Death Receptor; Flt-1, Fms-Related Tyrosine Kinase 1; GADD153, Growth Arrest and DNA Damage-Inducible 153; GRP78, Glucose-Regulated Protein; GSK3 β, Glycogen Synthase Kinase 3 β; HIF-1α, Hypoxia-Inducible Factor-1 α; HPV39, Human Papilloma Virus 39; HR, Homologous Repair; hTERT, Human Telomerase Reverse Transcriptase; hTR, Human Telomerase; HUVEC, Human Umbilical Vein Endothelial Cells; IFN, Interferon; IL, Interleukin; IκBβ, Inhibitor of Kappa B β; JNK, c-Jun N-Terminal Kinase; KDR/flk-1, Kinase Insert Domain Receptor; LC3, Microtubule-Associated Protein 1 Light Chain 3; MAPK, Nitogen-Activated Protein Kinase; MAX, MYC-Associated Factor X; Mcl-1, Myeloid Cell Leukemia 1; MDM2, Mouse Double Minute 2 Homologue; MEK, also known as MAPKK, Mitogen-Activated Protein Kinase Kinase; MMP, Matrix Metalloproteinase; MPNST, Malignant Peripheral Nerve Sheath Tumor; mTOR, Mammalian Target of Rapamycin; MYC, Avian Myelomastosis Viral Oncogene Homologue; NAC, N-Acetyl Cysteine; NFκB, Nuclear Factor Kappa B; NHEJ, Non-Homologous End-Joining; NO, Nitric Oxide; NOXA, Also Known As PMA/P1; Phorbol-12-Myristate-13-Acetate-Induced Protein 1; PARK7, Parkinson Disease Protein 7, Protein Deglycase DJ-1; PARP, Poly ADP Ribose Polymerase; PCNA, Proliferating Cell Nuclear Antigen; PGE2, Prostaglandine E2; PI3-K, Phospoinositide-3 Kinase; PMA, Phorbol-12-Myristate-13-Acetate; RAF, Ras-Associated Factor Proto-Oncogene; RAS, Rat Sarcoma Viral Oncogene Homologue; RKIP, Raf-1 Kinase Inhibitor Protein; ROS, Reactive Oxygen Species, SMAC/DIABLO, IAP-Binding Mitochondrial Protein; TCTP, Translationally Controlled Tumor Protein; TF, Transferrin; TFRC, Transferrin Receptor 1 Gene; TGB1, Triple Gene Block Protein β, TGF-beta, Tumor Growth Factor β, TIMP, Tissue Inhibitor of Metalloproteinase, TNF-α, Tumor Necrosis Factor α, TOPO2 A, DNA Topoisomerase 2 α, TRAIL, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand, Treg, Regulatory T Cells, VDAC2, Voltage-Dependent Anion Channel 2, VEGF, Vascular Endothelial Growth Factor, VEGFR, Vascular Endothelial Growth Factor Receptor, XIAP, X-Linked Inhibitor of Apoptosis, YY1, Yin Yang 1

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INTRODUCTION

Artemisinin is a sesquiterpenoid form Sweet Wormwood (*Artemisia annua* L., Asteraceae), which is known in Chinese medicine as *qinhao*. It was first described in the "Handbook of Prescriptions for Emergency Treatment" (*Hou Bei Ji Fang*, 肘后备急方) by Hong Ge (葛洪, (281-340 B.C.) to treat fever and chills. Remarkably, it was still included in the "Compedium of Materia Medica" (*Ben Cao Gang Mu*, 本草纲目), by Li Shizen (李时珍) more than one millennium later (1596). The fact that the medicinal use of this plant survived after such a long time may be already taken as a clue for the herb's activity.

It was China's former president, Mao Zedong, who started the secret research project No. 523 on May 23rd 1967 to identify a new malaria drug derived from Chinese medicine. The background was that during the Vietnam War a considerable portion of Vietnamese soldiers died from malaria. Therefore, the Vietnamese government asked China for a new anti-malaial drug. More than 500 scholars working in more than 60 laboratories screened the rich Chinese flora used in Chinese medicine. Among them, Youyou Tu investigated 640 out of 2000 traditional herbal mixtures. Although *A. annua* was the most active herb (No. 191, *qinhao*), the results were not reliably repeatable. Rather than standard procedures based on hot decoctions, the ancient texts mentioned that *A. annua* should used as the pressed juice. Youyou Tu discovered that low temperature extractions of *qinhao* provide the most effective preparations against malaria parasites[1-7]. The alteration of the extraction protocol brought the breakthrough, which ultimately led to the identification of the chemical structure of artemisinin (*qinhaosu*) in 1972.

Today, there is no doubt about that artemisinin (ARS) saved the lives of millions of people. ARS and its derivatives used as combination therapy together with other antimalarials belong to the standard treatments of malaria worldwide[8-14].

During the past few years, the full potential of Youyou Tu's discovery was recognized by the international scientific community. The conferment of the Lasker DeBakey Clinical Research Award and the Nobel Prize for Medicine or Physiology honors her lifetime achievements.[15-18] Youyou Tu and her team found early clues that the activity of ARS is not restricted to the treatment of malaria. Dihydroartemisinin inhibited the production of anti-ds-DNA antibodies, the secretion of TNF-α, and NF-κB signalling pathway. Thereby, dihydroartemisinin improved pathologic lesions associated with Lupus erythematosus-related nephritis *in vivo*[19-20].

MODE OF ACTION IN *PLASMODIA*

In the blood stream, *Plasmodium* trophozoites and schizonts reside inside the erythrocytes, where they feed on hemoglobin as the source for amino acids. Hemoglobin is toxic for the parasites. Heme-iron favors the generation of reactive oxygen species (ROS), which are detrimental to the parasites. Therefore, *Plasmodia* convert hemoglobin to the non-toxic hemozoin[21-22]. During hemoglobin digestion in the parasites' food vacuole, heme-iron is released, which facilitates the cleavage of artemisinin's endoperoxide bridge by a Fe(II) Fenton-type reaction. The transfer of an oxygen atom from the endoperoxide group of artemisinins to a chelated iron ion generates a Fe(IV)=O species. The resulting free radical intermediates then kill the parasites[23]. As a result, hydroxyl radicals and superoxide anions are formed that damage the food vacuoles of *Plasmodia* and lead to autodigestion[24-25]. Other mechanisms contributing to the inhibitory effects of ARS include

- the inhibition of redox cycling
- the inhibition of a glutathione S-transferase termed *Plasmodium falciparum* exported protein 1 (EXP1)
- the iron-mediated inhibition of *Plasmodium falciparum* PfATP6 orthologue, sarcoendoplasmatic reticulum Ca^{2+} ATPase (SERCA)
- the inhibition of digestive vacuole cysteine protease, as well as
- the alkylation of heme and several specific parasite proteins including translationally controlled tumor protein (TCTP)[26-31].

DNA damage has not been observed - an effect we refer to later in the context of cancer cells[32].

During the past years, it turned out that the bioactivity of ARS and its derivatives is much broader than initially thought. In addition to malaria, ARS-type drugs are also active against cancer *in vitro* and *in vivo*, certain viral infections (*e.g.* human cytomegalovirus, HCMV) infections, schistosomiasis *in vivo* and in patients, and even against plant tumors[33-38].

In the present review, we give a comprehensive and timely overview on the activity of ARS and its derivatives towards cancer cells *in vitro* and *in vivo* and give a perspective outlook on their clinical activity in tumor patients by reporting the present preliminary data from cancer patients. We only refer to ARS and the first generation derivatives, artesunate (ART), artemether (ARM), arteether (ARE), as well as the first metabolite, dehydroartemisinin (DHA). Second generation derivatives as well as nanotherapeutic approaches involving artemisinins are not considered here. Furthermore, combination treatment approaches between artemisinins and established or novel investigational compounds have also not been considered. To obtain a comprising overview of the published literature, we screened the PubMed database with the following search term combinatons: 'artemisinin/artesunate' and 'cancer' plus (1) '*in vivo*/xenograft/mice/rat', (2) 'cell cycle arrest', (3) 'reactive oxygen species/oxidative stress', (4) 'iron/transferrin', (5) 'DNA damage/DNA repair', (5) 'apoptosis/autophagy/necroptosis/ferroptosis', (6) 'angiogenesis/angiogenic', and (7) 'signaling/signal transduction'. The relevant literature has been considered until October 2015.

INHIBITION OF TUMOR CELL GROWTH *IN VITRO* AND *IN VIVO*

In the mid 1990s, two Chinese and three Western groups reported the activity of ARS and its derivatives in cancer cells

in vitro[39-42]. After these initial papers on selected tumor lines, a wealth of papers appeared in subsequent years, showing that ART and not only its main derivatives, ART, ARM, but also many new synthetic or semi-synthetically generated derivatives are able to kill cell lines of many different tumor types. Although the activity of these compounds largely varies from cell line to cell lines, there is overwhelming evidence that ARS-type drugs efficiently inhibit cancer cells. It is important to mention that the endoperoxide bridge plays a critical for bioactivity, since ARS-like compounds without this moiety do not display activity against *Plasmodia* or cancer cells[43-44].

The plethora of data on the *in vitro* cytotoxicity of ARS and its derivatives towards cancer cell lines, including stem-like cancer cells (for review see literature[33,45-48]) raised the interest on their antitumor activity *in vivo*.[33,45-48] Indeed, a number of studies demonstrated that this class of compounds was able to inhibit transplantable tumors in mice[49-74] (**Table 1**)

The majority of *in vivo* experiments with artemisinins have been performed with human xenograft tumors transplanted to immunocompromised athymic nude mice. Although this might appear as somewhat artificial approach for the investigation of antitumor activity, this is a well established and widely distributed animal model in drug research, as it allows to investigate response of human tumors to investigational novel drugs in living organisms without testing in human patients. The disadvantage that athymic mice lack an intact immune response may be overcome by the use of transplantable syngeneic murine tumors.

Remarkably, ARS, ART, and DHA demonstrated anticancer activity in both murine syngeneic and human xenograft tumors towards a wide range of different tumor types (**Table 1**). Hence, the cytotoxic activity of artemisinins towards cancer cell lines in numerous *in vitro* studies can be translated to the clinical situation. Rather, there is convincing evidence for the anticancer activity of ARS-type compounds in living organisms. Interestingly, the anticancer activity has not only been demonstrated in tumors subcutaneously transplanted, which is the standard procedure, but also in orthotopically transplanted tumors, which much better reflect the clinical situation in cancer patients.

INDUCTION OF OXIDATIVE STRESS

The cleavage of the endoperoxide bridge makes it probable that ROS are formed that contribute to the cytotoxic activity of this class of compounds. After unravelling the cytotoxic activity of ART towards cancer cells, we therefore tried to obtain mechanistic clues from mRNA microarray experiment, how artemisinins may reveal their cytotoxic activity. In a collaboration with the National Cancer Institute (NCI, USA), the $\log_{10}IC_{50}$ values to 55 cell lines derived from 8 different tumor types were determined. These results were correlated with the transcriptome-wide mRNA expressions in these cell lines and identified a number significant correlations between ART response and the expression of genes involved in cellular antioxidant response, *i.e.* antioxidative protein 2 (*AOP2*), catalase (*CAT*), dihydrodiol dehydrogenase (*DDH*), diaphorases (NADH/NADPH) cytochrome b-5 reductase (*DIA1, DIA4*), γ-glutamylcysteine synthetase (*GLCLR*), glutaredoxin 2 (*GLRX2*), glutathione S-transferases (*GSTA2, GSTM3, GSTM4, GSTT2, GSTZ1, MGST1, MGST3, MGST5*), glutathione peroxidases (*GPX1, GPX4*), oxidative stress response 1 (*OSM1*) manganese-dependent superoxide dismutase (*SOD1*), as well as thioredoxin peroxidase and reductase (*TXNPOX, TXNRD1*) [75-78]. These correlations were exemplarily verified by testing cell lines transfected with some of these antioxidant genes. WEHI7.2 cells transfected with cDNAs for *CAT*, *SOD1* or *TXN* and MSC-H13 cells transfected with *GLCLR* displayed resistance to ART compared to non-transfected or mock vector-transfected control cells[78-79]. Furthermore, small molecule inhibitors for γ-glutamylcysteine synthetase (*i.e.* buthionine sulfoximine, BSO) or glutathione S-transferases (*i.e.* ethacrynic acid, EA) were used to test the effect of these antioxidant proteins for ART response. Both BSO and EA sensitized MSC-H13 cells to ART, indicating that these antioxidant proteins confer ART resistance[78].

In subsequent years, a large body of evidence has been brought up confirming our initial results on the role of oxidative stress induced by artemisinins. ROS formation by ARS, ART or DHA has been reported in cell lines derived from many different cancer types, including hematopoietic tumors (leukemia, multiple myeloma, Non-Hodgkin lymphoma), mesenchymal tumors (embryonal rhabdomyosarcoma) and epidermal tumors (cancers of lung, liver, pancreas, colorectum, and cervix as well as melanoma) [57,73,77-96] (**Table 2**). The causative role of ROS for cytotoxicity has been shown by prooxidants (vitamins C and D3, dexamethasone) increasing the cell death rate and by antioxidants and ROS scavengers (N-acetyl-cysteine (NAC), vitamin E) suppressing artemisinin-induced cell death (**Table 2**).

ROLE OF IRON

Iron plays a crucial role for the cytotoxicity of artemisinins against cancer cells. Ferrous sulfate and holotransferrin increased DHA-induced cytotoxicity towards rat fibrosarcoma and breast carcinoma[97,98]. Ferrous iron in the form of iron(II)–glycine sulfate (Ferrosanol®) and holotransferrin increased the cytotoxicity of ARS, ART, and ART microencapsulated in maltosyl-beta-cyclodextrin towards CCRF-CEM leukemia and U373 astrocytoma cells as compared to drug application without iron[99]. Treatment of p53 wild-type TK6 and p53 mutated WTK1 lymphoblastic cells showed that mutational status of the tumor suppressor p53 did not influence sensitivity to ART. The effect of ferrous iron and transferrin was reversed by monoclonal antibody RVS10 against the transferrin receptor. This antibody competes with transferrin for binding to this receptor. CCRF-CEM and U373 cells expressed transferrin receptor in 95% and 48% of the cell population, respectively, whereas transferrin receptor

Table 1. Anticancer effect of artemisinins *in vivo*.

Tumor type	Cell line	Model type	Drug	Effect	Reference
Hepatic carcinoma	H22	Syngeneic	ART	Tumor growth↓, Bcl-2↓, Bax↑, PCNA↓	Wang et al., 2002
Ovarian carcinoma	HO-8910	Xenograft	ART	Tumor growth↓, VEGF↓, KDR/flk-1↓	Chen et al., 2004
Kaposi sarcoma	KS-IMM	Xenograft	ART	Tumor growth↓, vacularization of matrigel plugs↓	Dell'Eva et al. 2004
Oral mucosa tumor		Virally induced tumor	DHA	Formation of canine oral papillomavirus-induced tumors↓, antibody development against L1 capsid protein	Disbrow et al., 2005
Colorectal carcinoma		Xenograft	ART	Tumor growth↓, liver metastasis↓, Wnt/β-catenin pathway↓	Li et al., 2007
Hepatoma	HepG3, Hep3B	Xenograft	ART, DHA	Tumor growth↓, cyclin D1↓,cyclin E↓, CDK2↓, CDK4↓, Cip1/p21↑,Kip/p27↑, caspase-3↑, Bax/BCL-2 ratio↑, PARP↑,MDM2 ↓	Hou et al., 2008
Pancreas carcinoma	BxPC-3	Xenograft	DHA	Tumor growth↓, PCNA↓,cyclin D1↓, WAF1/C1P1↑, Bax↑, Bcl-2↓caspase-9↑,	Chen et al., 2009a; 2009b
Pancreas carcinoma		Xenograft	ART	Tumor growth↓	Du et al., 2009
Breast carcinoma	MTLn3	Syngeneic	ARS-TF	Tumor growth↓	Lai et al., 2009
Glioma	C6	Syngeneic, orthotopioc		Tumor growth↓, microvessel density↓	Wu et al., 2009
Breast cancer	MDA-MB-231	Xenograft	ART	Minimal inhibition due to resistance, NF-κB↑	Bachmeier et al., 2011
Breast cancer		Syngeneic	ART	Tumor growth↓, depletion of splenic CD4*, CD25+, Foxp3+ and Treg cells IL4↑, IFN-γ↑	Farsam et al., 2011
Leukemia	U937	Xenograft	DHA	Tumor growth↓, induction of apoptosis, ERK↓	Gao et al., 2011
Lung carcinoma	A549	Xenograft	ART	Tumor growth↓, induction of apoptosis, EGFR↓, AkT↓, ABCG2↓	Ma et al., 2011
Osteosarcoma	HOS	Xenograft	ART	Tumor growth↓, caspase-3↑	Xu et al., 2011
Breast cancer	MCF7	Xenograft	ARS	Tumor growth↓	Tin et al., 2012
Ovarian carcinoma	HO8910PM	Xenograft, orthotopic	DHA	Tumor growth↓, metastasis↓, CD31↓, pFAK↓, MMP2↓	Wu et al., 2012
Hepatocellular carcinoma		Xenograft	DHA	Tumor growth↓	Zhang et al., 2012
Osteosarcoma			DHA	Tumor growth↓, β-catenin↓, GSK3β↑	Liu et al., 2013
Gastric carcinoma	SGC 7901	Xenograft	DHA	Tumor growth↓, metastasis↓	Sun et al., 2013
Gastric carcinoma	BGC-823	Xenograft	ART	Tumor growth↓	Zhou et al., 2013
Murine mastocytoma	DBA2/P815	Syngeneic	ARS	Tumor growth↓	Tilaui et al., 2014
Hepatocellular carcinoma	HepG2, BWTG3	Xenograft		Tumor growth↓, microvessel density↓	Vandewynckel et al., 2014
Cervix carcinoma	HeLa, Hela/DHA	Xenograft	DHA	Inhibition of tumor growth more in sensitive HeLa than in DHA-resistant HeLa/DHA, over expression of DJ-1 (PARK7)	Zhu et al., 2014
Rat bladder carcinoma		Syngeneic, orthotopic		Tumor growth↓	Zuo et al., 2014

Table 2. Induction of oxidative stress by artemisinins.

Tumor type	Cell line	Drug	Effect	Reference
Diverse, Thymoma	55 NCI cell lines, WEHI7.2	ART	Correlation of microarray-based antioxidant gene expression with IC_{50} values. Transfection of antioxidant genes (thioredoxin, manganese superoxide dismutase, catalase) induced resistance to ART	Efferth et al., 2003; Efferth and Oesch, 2004
Diverse	50 NCI cell lines	ART	Correlation of 12 glutathione-related genes with IC_{50} values	Efferth and Volm, 2005
Leukemia	Jurkat, CCRF-CEM, CEM/ADR5000	ART	ROS↑, ROS scavening by NAC conferred ART resistance	Efferth et al., 2007
Non-Hodgkin lymphoma	Ramos	ART	ROS↑	Sieber et al., 2009
Pancreatic carcinoma	Panc-1, BxPC-3, CFPAC-1	ART	ROS↑	Du et al., 2010
Lung adenocarcinoma	ASTC-a-1	DHA	ROS↑	Lu et al., 2010
Hepatocellular carcinoma	HepG2	DHA	ROS↑	Gao et al., 2011
Melanoma	A375	DHA	Expression of oxidative and genotoxic stress response genes	Cabello et al., 2012
Pancreatic carcinoma	BxPC-3, PANC-1	DHA	ROS↑, ROS-mediated upregulation of death receptor DR5	Kong et al., 2012
Leukemia	K562	DHA	ROS↑	Wang et al., 2012
Lung adenocarcinoma	ASTC-a-1, A549	ART	ROS↑	Zhou et al., 2012
Lung carcinoma	A549		ROS↑	Gao et al., 2013
Lung carcinoma	A549	ART	ROS↑, ROS scavening by NAC confers ART resistance	Ganguli et al., 2014
Pancreatic carcinoma		DHA	ROS↑	Jia et al., 2014
Colorectal carcinoma	HCT-116	DHA	ROS↑	Lu et al., 2014
Pancreatic carcinoma	RIN	ARS	ROS↑	Noori et al., 2014
Multiple myeloma		ART	ROS↑	Papanikolaou et al., 2014
Cervical carcinoma	HeLa, HeLa/DHA	DHA	DJ-1 conferred DHA resistance by ROS removal	Zhu et al., 2014
Embryonal rhabdomyosarcoma	ERMS	ART	ROS↑, ROS-dependent expression of miR-133a and miR-206	Benefico et al., 2015
Leukemia	Molt-4	DHA	Prooxidants increasedcell death (vitamin C, vitamin D3, dexamethasone, H_2O_2). Antioxidants decreased cell death (vitamin E)	Gerhardt et al., 2015
Pancreatic carcinoma	PDAC	ART	ROS↑	Eling et al., 2015

expression in peripheral mononuclear blood cells of four healthy donors was confined to 0.4–1.3%. This indicates that artemisinins plus ferrous iron may affect tumor cells more than normal cells.

In addition to the transferrin receptor, specific ATP-binding cassette (ABC) transporters, *i.e.* ABCB6 and ABCB7, are also involved in iron homeostasis. To investigate whether these proteins play a role for sensitivity towards ART, Oncotest's 36 cell line panel was treated with ART or ART plus Ferrosanol®[100]. As expected, the majority of cell lines showed increased inhibition rates, for the combination of ART plus Ferrosanol® compared to ART alone. However, in 11 out of the 36 cell lines the combination treatment was not superior. Cell lines with high transferrin receptor expression significantly correlated with high degrees of modulation, indicating that high transferrin receptor-expressing tumor cells were more efficiently inhibited by this combination treatment than those with low transferrin receptor expression. In 55 NCI cell lines, a significant correlation was found between *ABCB6*, but not *ABCB7* mRNA expression and cellular response to ART. ART treatment of CCRF-CEM leukemia and MCF7 breast cancer cells induced *ABCB6* expression, but repressed *ABCB7* expression. Furthermore, ART inhibited proliferation and differentiation of mouse erythroleukemia (MEL) cells. Down-regulation of ABCB6 by antisense oligonucleotides inhibited differentiation of MEL cells indicating that ART and ABCB6 may cooperate. In conclusion, our results indicate that ferrous iron improves the activity of ART in some, but not all tumor cell lines. If it comes to the clinical application of ART for tumor treatment in the future, a general cotreatment with iron is rather not recommendable.

These initial data on the role of iron for the activity of artemisinins towards cancer cells have been corroborated by many publications in subsequent years[52,58,86,97-118] (**Table 3**). The iron chelator deferroxamine abolished the cytotoxicity of DHA, indicating a crucial role of iron for the activity of artemisinins. It was only recently, when the iron-dependent cytotoxicity of ARS-type compounds has been discussed in the context of a novel mode of cell death, termed ferroptosis (see below). The ferroptosis inhibitor ferrostatin-1 also inhibited DHA-induced cytotoxicity[118].

The correlation of iron homeostasis-regulating genes to the susceptibility of tumor cells raises the question, whether these genes might serve as biomarkers to predict the responsiveness of tumors to artemisinins in cancer patients. It is well known that the iron uptake is higher in highly proliferating tumors compared to normal tissues[119,120]. This may explain at least in part the preferential cytotoxicity of artemisinins towards tumor cells compared to normal cells. Cellular iron uptake and internalization are mediated by binding of transferrin–iron complexes to the transferrin receptor (CD71) expressed on the cell surface membrane and subsequent endocytosis. Transferrin receptor expression in normal tissues is limited to a few sites, *e.g.* basal epidermis, endocrine pancreas, hepatocytes, Kupffer cells, testis, and pituitary. Most other tissues do not express transferrin receptor[121]. In contrast, transferrin receptor is expressed in much larger amounts in proliferating and malignant cells [122-124] and it is widely distributed among clinical tumors[119-121]. It deserves further investigation, whether transferrin receptor and other iron-regulating genes and proteins may serve as biomarkers to predict the sensitivity of tumors to artemisinin-type drugs.

INDUCTION OF DNA DAMAGE AND REPAIR

Micorarray analyses on a panel of 60 NCI tumor cell lines revealed that the mRNA expression of several DNA damage response and repair genes significantly correlated with the $\log_{10}IC_{50}$ values of artemisinins for these cell lines, *e.g. ERCC5, FEN1, HMG1, HMF17, LIG1, RPS3, UNG,* and *UBE2A*[75,76]. Therefore, we hypothesized that ART may induce DNA damage due to the cleavage of the molecule's endoperoxide moiety, which may lead to ROS-or carbon-centered radical-mediated DNA damage.

Indeed, ART induced DNA breaks in a dose-dependent manner as shown by single-cell gel electrophoresis[125]. This genotoxic effect was confirmed by measuring the level of γ-H2AX, which is considered as marker for DNA double-strand breaks (DSB). Polymerase beta-deficient cells were more sensitive than the wild-type to ART, indicating that the drug induces DNA damage that is repaired by base excision repair. Irs1 and VC8 cells defective in homologous recombination (HR) due to inactivation of XRCC2 and BRCA2, respectively, were more sensitive to ART than the corresponding wild-type. This was also true for XR-V15B cells defective in nonhomologous end-joining (NHEJ) due to inactivation of Ku80. The data indicate that DSBs induced by ART are repaired by the HR and NHEJ pathways[125].

ART is a powerful inducer of oxidative DNA damage, giving rise to formamidopyrimidine DNA glycosylase-sensitive sites and the formation of 8-oxoguanine and $1,N^6$-ethenoadenine. Oxidative DNA damage was induced in human LN-229 glioblastoma cells together with apoptosic and necrotic cell death, which could be attenuated by radical scavengers such as N-acetyl cysteine (NAC). Oxidative DNA damage resulted in DSBs as determined by γ-H2AX foci. Upon chronic treatment with ART, DSBs continuously increased over the treatment period up to a steady-state level. This was in contrast to ionizing radiation, which induced a burst of DSBs followed by a decline due to their repair. Knockdown of Rad51 by siRNA and inactivation of DNA-PK strongly sensitized glioma cells to ART. These data indicate that both HJ and NHEJ pathways are involved in the repair of ART-induced DSBs. ART provoked a DNA damage response that was characterized by phosphorylation of ATM, ATR, Chk1, and Chk2.[126] Our initial findings on ART-induced DNA damage were confirmed for ARS, ARM, DHA, and ARS tagged to transferrin by other authors[75,76,125-130] (**Table 4**).

Table 3. Role of iron for the cytotoxicity of artemisinins towards cancer cells.

Tumor type	Cell line	Drug	Effect	Reference
Rat fibrosarcoma		DHA	Ferrous sulfate retarded tumor growth following DHA	Moore et al., 1995
Breast carcinoma		DHA	Holotransferrin increased cytotoxicity of DHA	Singh and Lai, 2001
Leukemia astrocytoma	CCRF-CEM , U373	ARS ART	Iron(II)-glycinesulfate (Ferrosanol®) and holotransferrin enhanced the cytotoxicity of artemisinins, while the monoclonal anti-transferrin receptor antibody RS10 decreased it.	Efferth et al., 2004
Leukemia	Molt-4	DHA	Holotransferrin increased cytotoxicity of DHA	Singh and Lai, 2004
Leukemia	Molt-4	ARS-TF	Transferrin tagging increased cytotoxicity of ARS	Lai et al., 2005a, 2005b
Cervical carcinoma	HCX-E6/E7, HeLa, SiHa, Caski	DHA	Transferrin receptor expression correlated with DHS sensitivity, iron-dependent ROS-formation	Disbrow et al., 2005
Diverse	36 cell lines, 55 NCI cell lines	ART	Ferrosanol® increased ART sensitivity in 25 out of 36 cell lines. IC_{50} values for ART correlated with the mRNA expression of TFRC and ABCB6 in 55 NCL cell lines	Kelter et al., 2007
Rat glioma	C6	ARS, DHA	Ferrous ions increased, deferoxamine abolished cytotoxicity.	Lu et al., 2008
Rat breast tumor	MTLn3	ARS-TF	Inhibition of tumor growth, no side effects	Lai et al., 2009
Prostate carcinoma	DU145	ARS-TF, ART-TF	The conjugates retained activity of untagged ARS. siRNA-mediated knockdown of transferrin impaired ART-transferrin, but not ARS-transferrin	Nakase et al., 2009
		ARS	Heme (Fe^{2+} protoporphyrin IX) increased cytotoxicity	Zhang and Gerhard, 2009
Breast carcinoma	MCF-7	ART	Iron induced mitochondrial apoptosis, deferoxamine abolished cytotoxicity.	Hamacher-Brady et al., 2011
Colorectal carcinoma	HCT-116	DHA	Iron-dependent endoplasmic reticulum stress. GRP78↑, GADD153↑,deferoxamine aboslished these effects	Lu et al., 2011
Cervical carcinoma	HeLa	ARS	Heme and holotransferrin enhanced endoperoxide activation and cytotoxicity.	Mercer et al., 2011
		DHA	DHA depleted cellular iron and down-regulated transferrin receptor expression by a lipid raft-mediated internalization pathway	Ba et al., 2012
Leukemia	K562	DHA	Iron-loaded cells underwent autophagy downregulation of transferrin-receptor expression	Wang et al., 2012
Hepatoblastoma, Hepatocarcinoma colon carcinoma	HepG2, SK-HEP1, LS174T	ARS and others	Ferrosanol®, but not hemin increased cytotoxicity	Blazquez et al., 2013
Leukemia	Molt-4		Deferoxamine attenuated cytotoxicity of DHA	Chan et al., 2013
Hepatocellular carcinoma	SMMC-7721	ARS	Holoferrin enhanced the cytotoxic activity of ARS	Deng et al., 2013
Retinoblastoma	RB-Y79	ART	ART internalization was dependent upon transferrin receptor	Zhao et al., 2013

Table 3. (Continued)

Tumor type	Cell line	Drug	Effect	Reference
			expression, siRNA-mediated knockdown of transferrin receptor decreased ART	
Canine histiocytic sarcoma	DH82	DHA	Endophagic uptake of heme-iron enhanced DHA cytotoxicity, suggesting a role of exogenous heme	Chikazawa et al., 2014
Hepatocellular carcinoma, lung adenocarcinoma	HepG2, A549	ARS, ART, DHA	Binding to transferrin enhanced cellular uptake	Yang et al., 2014
Renal cell carcinoma	Caki-1, 786-0, SN12C-GFP-SRLu2	ART	Transferrin receptor expression is correlated with metastasis and unfavorable prognosis. ART cytotoxicity correlated with transferrin receptor expression	Jeong et al., 2015
Diverse	55 NCI cell lines	DHA, ARS, ART, ARE, ARM and others	mRNA expression of 20 iron-regulating genes correlated with IC_{50} values of artemisinins. Ferrostatin and deferoxamine abolished DHA-cytotoxicity in CCRF-CEM cells.	Ooko et al, 2015

INDUCTION OF CELL CYCLE ARREST

It can be expected that ROS generation and oxidative DNA damage massively disturb cellular integrity, which affects the basic cellular machinery involved in replication and cell division. In cancer biology, it is common sense that DNA damage induced by anticancer agents cause cell cycle arrest and apoptosis. Hence, it comes as no surprise that numerous investigations described cell cycle arrest upon treatment of tumor cells with artemisinin-type compounds[54-56,64-65,67,69,76,91,108,114,117,131-150] (**Table 5**). Again, this phenomenon has been observed, independently as to whether the cell lines derived from hematopoietic, mesenchymal or epidermal origin. It may be surprising, however, that the halt of cell cycle progression does take place both at G1 and G2 checkpoints. As these G1 or G2 arrests do not seemingly occur in a tumor-type or drug-specific fashion, individual aberrations in the cell cycle machinery may determine, whether a cell line rather induces G1 or G2 cell cycle arrest upon exposure to artemisinins.

A panel of tumor cell lines treated under comparable conditions (the same conditions of maintenance, the same detection method, the same experimentator etc.) showed that three of 7 cell lines induced G1 arrest, while others arrested the cell cycle in G2[138]. This reflects the general situation documented in the literature (**Table 5**).

Considering the paramount importance of the p53 pathway for G1 arrest, p53 and $p21^{WAF1/CI/P1}$ were analyzed in more detail and used human wild-type HCT-116 colon cancer cells ($p53^{+/+}$ and $p21^{WAF1/CIP1+/+}$) and isogenic knockout clones ($p53^{-/-}$, $p21^{WAF1/CIP1-/-}$ and $p53^{-/-}/p21^{WAF1/CIP1-/-}$)[76]. The incorporation of bromodeoxyuridine (BrdU) was inhibited in all three cell lines in a time-dependent manner and to a similar extent. This indicates that the two knockout cell lines were similarly sensitive to ART-induced inhibition of proliferation as wild-type HCT-116 cells. Using immunoblotting and kinase assays, the protein expression and kinase activity of cell cycle regulating genes were analyzed in wild-type cells and knockout mutants. Treatment

Table 4. Induction of DNA damage and repair by artemisinins in cancer cells.

Tumor type	Cell line	Drug	Effect	Reference
Diverse	60 NCI cell lines	ART, ARE, ARM,	The mRNA expression of genes related to DNA damage and repair correlated to IC_{50} of artemisinins	Efferth et al., 2001; 2002; 2003
		ART	Induction of DNA double-strand breaks. Involvement of base excision repair, homologous repair (HR) and non-homologous end-joining (NHEJ) in ART induced DNA damage.	Li et al., 2008
		ART	Induction in oxidative DNA damage that results in DNA-double strand breaks. Involvement of HR and NHEJ	Berdelle et al., 2011
Gastic carcinoma	PG100	ARM	Induction of DNA damage	Alcântara et al., 2013
Hepatocellular carcinoma		ARS ART	Induction of DNA damage	Aquino et al., 2013
Leukemia	MOLT-4, RTN	DHA, ART-TF	DHA-resistant RTN cells revealed less X-ray-induced DNA damage than wild-type Molt-4 cells	Park et al., 2015

Table 5. Cell cycle effects of artemisinins in cancer cells.

Tumor type	Cell line	Drug	Effect	Reference
Diverse	55 NCI cell lines	ART	Correlation of G_0G_1 and S phases to IC_{50}	Efferth et al., 2003
Ovarian carcinoma		DHA	G_2M phase arrest	Jiao et al., 2007
Breast cancer	MCF7	ARS	G_0G_1 phase arrest	Sundar et al., 2008
Hepatoma		ART, DHA	G_0G_1phase arrest; cyclin D1↓, cyclin E↓, CDK2↓, CDK4↓, E2F1 ↓, p21 ↑, p27↑	Hou et al., 2008
Leukemia	K562	ART	G_0G_1 phase arrest	Yao et al., 2008
Prostate carcinoma	PC-3	ART	G_2M phase arrest	Huang et al., 2008
Pancreatic carcinoma	BxPC-3, AsPC-1	DHA	G_0G_1phase arrest; regulation of cyclin E, CDK2↓, CDK4↓, p27↑, p21↑	Chen et al., 2009a, 2009b, 2010
Multiple myeloma	SP2/0	ART	G_0G_1 phase arrest	Li et al., 2009
Lymph node carcinoma of the prostate	LnCaP		G_0G_1 phase arrest; CDK2↓, CDK4↓, pSp1 ↓	Willoughby et al., 2009
Leukemia	CCRF-CEM, CEM/ADR5000	Artesunic acid	G_0G_1 phase arrest	Horwedel et al., 2010
		ART		Steinbrück et al., 2010
Osteosarcoma		DHA	G_2M phase arrest; cyclin D1↑, CDC25B↓, cyclin B1↓	Ji et al., 2011
Colorectal carcinoma	HCT116	DHA	G_0G_1 phase arrest	Lu et al., 2011
Nasopharyngeal carcinoma	CNE-1, CNE-2	ARS	G_0G_1 phase arrest; p16↓, CDK4↓	Wu et al., 2011
Osteosarcoma	HOS	ART	G_2M phase arrest	Xu et al., 2011
Epidermoid carcinoma	A431	ART	G_0G_1 phase arrest; cyclin A1↓, cyclin B1↓, cyclin D1↓, CDK2↓, CDK4↓, CDK6↓	Jiang et al., 2012
	GH3	ART	G_2M phase arrest	Mao et al., 2012
Breast cancer	MCF7	ARS	G_0G_1 phase arrest; cyclin D1↓, cyclin E↓, CDK2↓, CDK4↓, E2F1↓	Tin et al., 2012
Hepatocellular carcinoma		DHA	G_2M phase arrest; p21↑, CDC25B↓, cyclin B↓	Zhang et al., 2012
Endometrial carcinoma	RL95-2	ART	G_0G_1 phase arrest	Zheng et al., 2012
Esophageal carcinoma		DHA	G_0G_1 phase arrest; cyclin E↓, CDK2↓, CDK4↓	Du et al., 2013
Breast carcinoma		DHA	G_0G_1 phase arrest	Mao et al., 2013
Gastric carcinoma	SGC-7901, BGC823, MGC803	DHA	G_0G_1phase arrest, p21↑, p27↑, PCNA↓, cyclin E↓, cyclin D1↓	Sun et al., 2013
Retinoblastoma	RB-Y79	ART	G_0G_1 phase arrest	Zhao et al., 2013
Glioma	Stem cells	DHA	G_0G_1 phase arrest	Cao et al., 2014
Breast cancer	MCF7, MDA-MB-231	ART	G_2M phase arrest, p21↑	Chen et al., 2014
Colorectal carcinoma	HCT116, HCT116/R	DHA	G_0G_1 phase arrest, GADD153↑, GRP78↑	Lu et al., 2014
Endometrial carcinoma	Ishikawa	ARS	G_0G_1 phase arrest; CDK2↓, CDK4↓	Tran et al., 2014
Gastric carcinoma	AGS, MKN74	ARS	p21↑, p27↑	Zhang et al., 2014
Neuroblastoma		ARS	G_0G_1 phase arrest	Zhu et al., 2014
Renal cell carcinoma	Caki-1, 786-O, SN12C-GFP-SRLu2	ART	G_2M phase arrest	Jeong et al., 2015

with ART induced the p53 protein expression in wild-type cells but not in p53 and $p21^{WAF1/CIP1}$ knockout cells. The $p21^{WAF1/CIP1}$ protein was strongly induced in wild-type cells and very weakly induced in p53/$p21^{WAF1/CIP1}$ knockout cells upon ART treatment. Hypophosphorylation of the tumor suppressor protein RB coincided with a down-regulation of *CDK2* kinase activity in response to ART treatment, which is indicative of G1 arrest. Protein expression and kinase activity of the G2/M regulator cyclin B1 declined after treatment of all three cell lines with ART[76]. Furthermore, the conditional expression of the *CDC25A* gene using a tetracycline repressor expression vector increased ART sensitivity[76]. This speaks for a role of ART in arresting cells in the G1 phase.

Cells residing in the G2/M arrest revealed multiple centrosomes, small multiple spindles and multi-nucleated cells, suggesting a defect in cytokinesis. The mitotic spindle checkpoint genes *bub1, bub2, bub3, mad1, mad2* and *mad3* were individually deleted and the sensitivity of these mutants towards ART was determined by monitoring the cell growth. The *Δbub3* and *Δmad3* mutants showed an increased

sensitivity and the Δmad2 mutant a slightly decreased sensitivity to ART in comparison to the respective wild type. The Bub3, Mad3 and Mad2 proteins are the main regulators of the mitotic spindle checkpoint, suggesting that ART may interfere with this control mechanism[127,138].

G1 arrest induced by ARS, ART or DHA was accompanied by specific changes in the expression of cell cycle-regulating genes/proteins, *e.g.* down-regulation of cyclins A1, D1 and E, CDKs 2, 4 and 6, and up-regulation of p21 and p27 and others. On the other hand, arresting the cell cycle in G2 by artemisinins was associated with down-regulation of cyclin B and CDC25B and up-regulation of cyclin D1. These data speak for the specificity of cell cycle blockage and the controlled regulation, whether G1 or G2 arrest is induced after treatment of tumor cells with artemisinins.

INDUCTION OF CELL DEATH

Apoptotic cell death

Oxidative stress and DNA damage not only provoke cell cycle arrest and DNA repair, but also ultimately lead to cell death. In 1996, Efferth et al. were the first to describe that ART induces apoptosis in tumor cells[42] - a result that has been confirmed by numerous subsequent publications in the following years[42,52,54-56,60,62-64,67-69,71,74-76,80-84,87-89,91-93,101,104,108,131,132,134,135,139,141,46,151-178] (**Table 6**). Later on, ART was found to induce both the intrinsic, mitochondrial as well as the extrinsic FAS-receptor-driven pathway of apoptosis[80,81] with induced Fas/CD95 expression, breakdown of the mitochondrial membrane potential, cytochrome C release, PARP cleavage and caspase 3/9 activation. Bcl-2 transfected cells were more resistant to artesunate[79]. In the meantime, a mass of results are available for cell lines inducing either the intrinsic or extrinsic pathway of apoptosis upon challenge with artemisinins (**Table 6**).

Non-apoptotic cell death

In addition to caspase-dependent apoptosis, artemisinins are also able to induce non-apoptotic forms of caspase-independent cell death[70,86,90,93,96,107,118,128,144,47,179-181] (**Table 7**). In 2011, the induction of autophagy by ART was reported[107], which was corroborated by other authors later on (**Table 7**). Autophagy represents a cellular emergency mechanism in response to the nutrient depletion, damaged organelles or other cellular stress situations. For properly degradation and recycling, cellular compoents are engulfed in autophagosomal vesicles, which are transported to lysosomes, where the degradation takes place. A key player in the autophagy process is mTOR1, which activates the ULK1 kinase complex (*ULK1*, *ATG13*, *ATG17*) leading to autophagosome formation. Depending on the cellular context, recycling of cellular material by autophagy may lead either to improved cell survival or cell death.

The role of necrosis and necroptosis as relevant modes of cell death for artemisinins has been emphasized too (**Table 7**). While necrosis is understood as accidental and non-programmed cell death, necroptosis shares features of necrosis, but occurs in a programmed fashion. Necroptosis (or inflammatory cell death) represents a cellular defense mechanism against viral or other microbial attack.

Another related form of accidental or passive cell death is oncosis (ischemic cell death), which is characterized by cytosolic vacuolization as well as swelling of mitochondria, nucleus and cytoplasm. A few authors reported oncosis in response to treatment of cancer cells with artemisinins[57,70].

Recently a specific novel mode of iron-dependent cell death, termed ferroptosis has been unraveled[182]. Given the crucial role of iron for the cytotoxic action of artemisinins, the involvement of ferroptosis is obvious (**Table 7**). This is a novel type of caspase-independent non-apoptotic cell death, which is dependent on the intracellular presence of iron.[183] In ferroptosis, RAS-mutated tumor cells commit programmed cell death with concomitant increases of ROS levels and decreases of mitochondrial sizes. The exact mechanism of ferroptosis is yet to be clarified. Intracellular cysteine import mediated by a glutamate-cysteine-antiporter system in the cell membrane suppresses ferroptosis. Cysteine is needed for the synthesis of glutathione and glutathione prevents the accumulation of lipid peroxides. Ferroptosis occur by inhibition of glutathione peroxidase 4. Erasin, an oncogenic RAS-selective lethal compound, as well as the kinase inhibitor sorafenib inhibited the cysteine–glutamate antiporter complex x_{c-} and induced ferroptosis[182-183]. Ferrostatin-1 and deferoxamine are iron-depleting agents that inhibit ferroptosis[184-185].

ART specifically induced ROS- and lysosomal iron-dependent ferroptosis in KRAS-mutant pancreatic ductal adenocarcinoma cell lines with constitutively active K-RAS[96]. Ferrostatin-1 blocked ART-induced lipid peroxidation and cell death. Analysis of mRNA microarray data of pancreatic carcinoma showed a dependency on antioxidant homeostasis and increased sensitivity to free intracellular iron, both of which correlated with RAS-driven sensitivity to ferroptosis.

Ooko et al. (2015) correlated the $\log_{10}IC_{50}$ values of 10 artemisinin derivatives to the microarray-based mRNA expression of 30 iron-related genes in 60 NCI cell lines as determined in 218 different microarray hybridization experiments[118]. The mRNA expression of 20 genes represented by 59 different cDNA clones significantly correlated to the $\log_{10}IC_{50}$ values for the artemisinins, including genes encoding transferrin (TF), transferrin receptors 1 and 2 (*TFRC, TFR2*), cerulopasmin (*CP*), lactoferrin (*LTF*) and others. Ferrostatin-1 and deferroxamine reduced the cytotoxicity of DHA. Pre-therapeutic determination of iron-related genes may indicate tumor sensitivity to artemisinins. Ferroptosis induced by ARS-type drugs deserve further investigation for individualized tumor therapy.

INHIBITION OF ANGIOGENESIS

Natural products act in a rather multi-target specific manner compared to targeted synthetic small molecule inhibitors[186]. From an evolutionary point of view, it makes much more sense for plants to have broad-spectrum and versatile chemical

Table 6. Induction of apoptotic cell death by artemisinins in cancer cells.

Tumor type	Cell line	Drug	Effect	Reference
Leukemia	KG-1a	ART	Apoptosis	Efferth et al., 1996
Diverse	55 NCI cell lines	ART, ARE, ARM	Correlation of microarray-based apoptosis-regulating genes to IC_{50} values,p53-independent apoptosis.	Efferth et al., 2002; 2003
Leukemia	Molt-4	ARS	Induction of apoptosis, but not necrosis	Singh and Lai, 2004
Oral squamous cell carcinoma	IHGK	ARS	Induction of apoptosis, Bax↑, Bcl-2↓	Yamachika et al., 2004
Cervical carcinoma	HCX-E6/E7, HeLa, SiHa, Caski	DHA	P53-independent apoptosis, caspase 9↑, PARP↑,	Disbrow et al., 2005
Leukemia	Jurkat, CCRF-CEM	ART	Intrinsic pathway of apoptosis	Efferth et al., 2007
Rat glioma	C6	DHA	Induction of apoptosis, HIF-1 α↓	Huang et al., 2007
Ovarian carcinoma		DHA	Induction of apoptosis Bcl-2↓, Bcl-xL↓, Bax↑, Bad↑	Jiao et al., 2007
Rat glioma	C6	DHA	Induction of apoptosis	Ma et al., 2007
Lung cancer	SPC-A-1	DHA	Induction of apoptosis, survivin↓	Mu et al., 2007
Lung carcinoma	PC-14	DHA	Induction of apoptosis, Ca^{2+}↑, p38 activation	Mu et al., 2008
Leukemia	U937	ART	Induction of apoptosis, induction of T-cell mediated dendritic antileukemic responses *in vitro*	Zheng et al., 2007
Canine osteosarcoma	OSCA2, OSCA16, OSCA50, D17	DHA	Induction of apoptosis, caspase 3↑	Hosoya et al., 2008
Hepatoma	HepG2, Huh-7, BEL-7404, Hep3B	ART, DHA, ARM, ARS	Induction of apoptosis, Bax/Bcl-2 ratio↑, PARP↑, MDM2↓	Hou et al., 2008
Leukemia	HL-60	DHA	Induction of apoptosis, p38 MAPK↓	Lu et al., 2008
Leukemia	K562	DHA	Induction of apoptosis CHK↑, DNA-PK↓, TOPO1↓, MCL-1↓	Yao et al., 2008
Pancreatic carcinoma	BxPC-3, AsPC-1	DHA	Induction ofapoptosis, nuclear NF-κB p65↓, Bax↑, Bcl-2↓,caspases 3/9↑	Chen et al., 2009a; 2009b; 20010
Multiple myeloma	SP2/0	ART	Induction of aooptosis, nuclear NF-κB p65↓, IkBβ↑	Li et al., 2009
Lung adenocarcinoma	ASTC-a-1	DHA	Induction of apoptosis, mitochondrial membrane potential↓, caspase 3↑	Lu et al., 2009
Melanoma		ART	Induction of apoptosis in melanoma cells of ret-transgenic mice	Ramacher et al., 2009
Non-Hodgkin lymphoma	Ramos	ART	Extrinsic pathway of apoptosis, YY1↓, Sp1↓, Bid↑	Sieber et al., 2009
Pancreatic carcinoma	MiaPaCa-2, BxPC-3	ART	Induction of apoptosis, caspases 3/7↑, TOPO2A↓	Youns et al., 2009
Leukemia	Raji, Jurkat, ALL primary cells		Induction of apoptosis, mitochondrial membrane potential↓, caspase-3↑	Zeng et al., 2009
Murine lung carcinoma	Lewis	DHA	Induction of apoptosis	Zhou et al., 2009
Leukemia	Jurkat	DHA	Induction of apoptosis, mitochondrial membrane potential↓, cytochrome C release, caspases↑, Bcl-2↓, Bcl-cL↓, NOXA↑,Bax↑	Handrick et al., 2010
Prostate carcinoma		DHA	Induction of intrinsic and extrinsic apoptosis, P13-K/AKT and ERK pathways↓,death receptor DR5↑	He et al., 2010
Lung adenocarcinoma	ASTC-a-1	DHA	Induction of intrinsic and extrinsic apoptosis, mitochondrial membrane potential↓, cytochrome C release, caspases 3/8/9↑, Bid↑	Lu et al., 2010
Neuroblastoma	16 cell lines	ART	Induction of apoptosis, role of glutathione mechanism	Michaelis et al., 2010
Breast cancer	MCF-7, MDA-MB-231	ART	Induction of apoptosis; resistance by NF-κB↑, Bcl-2 ↑ and Bax↓	Bachmeier et al., 2011
Pancreatic carcinoma	BxPC-3	DHA	Induction of apoptosis, Bcl-2↓, Bax↑	Aung et al., 2011
Leukemia	AML and ALL primary cells	DHA	Induction of apoptosis, ctochrome C release, caspase↑, Mcl-1↓,MEK/ERK↓	Gao et al., 2011

Table 6. (Continued)

Tumor type	Cell line	Drug	Effect	Reference
Hepatoma	HepG2	DHA	Induction of apoptosis, Ca^{2+}↑, GADD153↑,BaX↑,Bcl-2↑	Gao et al., 2011
Osteosarcoma		DHA	Induction of intrinsic and extrinsic apoptosis, caspases 3/8/9↑, FAS↑,Bax↑, Bcl-2↓, NF-κB↓	Ji et al., 2011
Colorectal carcinoma	HCT-116	DHA	Induction of apoptosis endoplasmic reticulum stress, GRP78↑, GADD153↑	Lu et al., 2011
Lung carcinoma	A549	ART	Induction of apoptosis, EGFR↓, AKT↓, ABCG2↓	Ma et al., 2011
Cervical carcinoma	HeLa	ART	Induction of extrinsic apoptosis, survivin↓, XIAP, AKT inactivation, inhibition of TRAIL-induced transcriptional activation of NF-κB	Thanaketpaisarn et al., 2011
Osteosarcoma	HOS	ART	Induction of intrinsic apoptosis, cytochrome C release, Bax↑, Bcl-2↓,caspases 3/9↑	Xu et al., 2011
Metastatic melanoma	A375, G361, LOX	DHA	Induction of apoptosis, p53 phosphorylation, NOXA↑	Cabello et al., 2012
Lung adenocarcinoma	A549, ASTC-a-1	DHA	Induction of apoptosis, induction of endoplasmic reticulum stress, Bim↑	Chen et al., 2012
Leukemia	K562	DHA	Induction of apoptosis BCR/ABL↓	Gao et al., 2012
Epidermoid carcinoma	A431	ART	Induction of intrinsic apoptosis	Jiang et al., 2012
Colorectal carcinoma	HCT-116/R	DHA	Induction of apoptosis, heat shock proteins↑	Lu et al., 2012
Prostate cacinoma	PC-3M		Induction of apoptosis, caspases 3/8↑	Wang et al., 2012
Hepatocellular carcinoma		DHA	Induction of apoptosis,catochrome C release, caspases 3/9↑, Mcl-1↓, NOXA↑,Bax↑	Zhang et al., 2012
Lung adenocarcinoma	ASTC-a-1, A549	ART	Induction of intrinsic apoptosis , release of Smac and AIF, Bak↑, VDAC2↓,Bim↑	Zhou et al., 2012
Esophageal carcinoma		DHA	Induction of apoptosis, Bax↑, Bcl-2↑, Bcl-xL↓,procaspase-3↓, caspase-9↑	Du et al., 2013
Lung cancer	A549	ARS	Induction of apoptosis, mitochondrial membrane potential↓, Bid cleavage,release of SMAC and AIF, caspases 3/8/9↑	Gao et al., 2013
Multiple myeloma, diffuse large B-cell lymphoma		ART	Induction of apoptosis, MYC↓, Bcl-2↓, caspase 3↑	Holien et al., 2013
Nasopharyngeal carcinoma	CNE-2	DHA	Induction of apoptosis, caspase 3↑	Huang et al., 2013
Leukemia	CML cells	DHA	Induction of apoptosis, BCR/ABL↓, AKT↓, ERK↓, cytochrome C release, caspases 3/9↑	Lee et al., 2013
Osteosarcoma		DHA	Induction of apoptosis, GSK3β↑	Liu et al., 2013
Breast cancer		DHA	Induction of intrinsic apoptosis, cytochrome C release, caspases 8/9↑, Bid activation, Bim↑, Bcl-2↓	Mao et al., 2013
Pancreatic carcinoma	R/N	ARS	Induction of apoptosis	Noori et al., 2014
Gastric carcinoma	SGC-7901, BGC823, MGC803	DHA	Induction of apoptosis, Bcl-2↓, caspase 9↑, PARP↑	Sun et al., 2013
Glioma	Stem cells	DHA	Induction of apoptosis, p-AKT↓, caspase 3↑	Cao et al., 2014
Lung cancer, squamous cell carcinoma, breast carcinoma	A549 , SCC25, MDA-MB-231	ART	Induction of apoptosis and autophagy, accumulation of acidic vacuoles, cytochrome C release, caspase 3↑	Ganguli et al., 2014
Cervical carcinoma	HeLa, Caski	DHA	Induction of apoptosis, RKIP↑, Bcl-2 ↓	Hu et al., 2014
Colorectal carcinoma		DHA	Induction of apoptosis, mitochondrial membrane potential↓, caspases 3/8/9↑, cytochrome C release, AIF translocation	Lu et al., 2014
Rhabdomyosarcoma	Rh30, RD	DHA	Induction of apoptosis	Odaka et al., 2014
Multiple myeloma		ART	Induction of non-caspase apoptosis, mitochondrial membrane potential↓, translocation of A/F and EndoG	Papanikolaou et al., 2014
Murine mastocytoma, hamster kidney adenocarcinoma	P815, BSR	ARS	Induction of apoptosis	Tilaouri et al., 2014
Bladder cancer		ART	Induction of apoptosis, miR-16↑, COX-2↓, PGE2↓	Zuo et al., 2014
Osteosarcoma	143B	DHA	Induction of apoptosis	Liu et al., 2015
HPV-39 infected cervical carcinoma	ME-180	ARS	Induction of apoptosis, decreased telomerase activity, hTR↓, hTERT↓,HPV-39 E6 and E7↓	Mondal and Chatterji, 2015
Gastric cancer		ART	Induction of intrinsic apoptosis, COX2↓, Bax↑, Bcl-2↓,mitochondrial membrane potential↓, caspases 3/ 9↑	Zhang et al., 2015

Table 7. Induction of non-apoptotic cell death by artemisinins in cancer cells.

Tumor type	Cell line	Drug	Effect	Reference
Pancreatic carcinoma	Panc-1, BxPC-3, CFPAC-1	ART	Induction of oncosis, depolarization of mitochondrial membrane	Du et al., 2010
Breast cancer	MCF-7	ART	Induction of autophagy, inhibition of autophagosome turnover, perinuclear clustering of autophagosomes, early and late endosomes and lysosomes	Hamacher-Brady et al., 2011
Leukemia	K562	DHA	Induction of autophagy, LC3-II↑	Wang et al., 2012
Gastric cancer	PG 100	ARM	Induction of necrosis	Alcântara et al., 2013
Gastric cancer	SCG-7901, BCG-823, AGS	ART	Induction of oncosis, rahther than apoptosis	Zhou et al., 2013
Schwannoma	RT4	ART	Induction of necroptosis	Button et al., 2014
Breast carcinoma	MCF7, MDA-MB-231	ART	Induction of autophagy, beclin1↓, stimulation of LC3 stimulation, p21↑	Chen et al., 2014
Diverse		DHA	Induction of autophagy, NF-κB↓	Hu et al., 2014
Pancreatic carcinoma		DHA	Induction of autophagy, beclin1↑, JNK pathway↑	Jia et al., 2014
Multiple myeloma		ART	Induction of non-caspase apoptosis, depolarization of mitochondrial membrane, translocation of AIF and EndoG	Papanikolaou et al., 2014
Diverse		DHA	Induction of autophagy by p8 endoplasmic reticulum stress-related ATF4 and CHOP↑	Chen et al., 2015
Pancreatic carcinoma	PDAC	ART	Induction of ferroptosis	Eling et al, 2015
Diverse	60 NCI cell lines	ART, ARS, ARE, ARM	Correlation of iron-regulating genes with IC_{50} values of artemisinins	Ooko et al., 2015

weapons in their armamentory to defense themselves from microbial attack or herbivores[187]. Mono-specific drugs gained interest in the past years in pharmacology to decrease unwanted side effects and potentially increase therapeutic effects on disease-related targets in human patients. In nature, mono-specific compounds may be inferior due to rapid resistance development – a phenomenon that is also well known in pharmacology and which represents a major obstacle in cancer and many infectious diseases.

Therefore, it is probable that artemisinins also act against cancer cells by multiple mechanisms. A number of publications provided evidence that artemisinins inhibit angiogenesis[50,51,59,72,161,165,170,177,188-198] (**Table 8**). This has been shown by using blood vessel endothelial cells (HUVEC), chicken eggs and the corioallantoic membrane (CAM) assay *in vivo* as well as animal models using matrigel plugs or xenograft tumors. The secretion of angiogenic factors (*e.g.* VEGF, KDR/flk-1, VEGFR2) by tumor cells is inhibited by ARS treatment.

ART not only inhibited the growth of HUVEC cells *in vitro*, but also angiogenesis and *in vivo* growth of a human Kaposi sarcoma xenograft, which had been established from a renal transplant patient with a Kaposi sarcoma lesion[51]. Furthermore, ART also strongly reduced angiogenesis *in vivo* regarding the vascularization of matrigel plugs subcutaneously injected into syngeneic mice[51].

The mRNA expression data of 89 angiogenesis-related genes obtained by microarray hybridization from the NCI database were compared with the $\log_{10}IC_{50}$ values for 8 artemisinins (ARS, ARE, ART, ARM, artemisetene, arteanuine B, dihydroartemisinylester stereoisomers 1 and 2). The constitutive expression of 30 genes correlated significantly with the cellular response to these compounds. By means of hierarchical cluster analysis and cluster image mapping expression, profiles were constructed that significantly determined the cellular response to ART, ARE, ARM and dihydroartemisinylester stereoisomer 1. The microarray data of six out of these 30 genes were exemplarily validated by real-time RT-PCR in seven cell lines. The fact that sensitivity and resistance of tumor cells could be predicted by the mRNA expression of angiogenesis-related genes. This strongly indicates that inhibition of angiogenesis represents an important mode of action of artemisinins in tumors.

To further investigate the anti-angiogenic potential of artemisinins, *in vivo* experiments were performed in a Zebrafish model and subjected the results to molecular docking and quantitative structure relationship (QSAR) analyses[199,200]. A statistically significant inverse relationship was obtained between *in silico* binding energies to vascular endothelial growth factor receptor 1 (VEGFR1) and angiogenic activity *in vivo*. This data set was used as control experiment to validate molecular docking to predict angiogenic activity. Then, 52 artemisinin derivatives were docked to VEGFR1, VEGFR2, and VEGFA. The best binding affinities were found for VEGFR1. Using a combined docking/QSAR approach, candidate compounds were identified for further analysis[200].

Table 8. Inhibition of angiogenesis by artemisinins.

Tumor type	Cell line	Drug	Effect	Reference
Endothelial cells	HUVEC	ART	migration in scratch assay↓, microvessel tube-like formation on collagen gel↓	Chen et al., 2003
		ART	blood vessel formation *in vivo* using the matrigel plug assay↓	Dell-Eva et al., 2004
Ovarian carcinoma,	HO-8910 (*in vivo*)	ART	tumor growth↓, VEGF↓, KDR/flk-1↓,	Chen et al., 2004a
Endothelial cells	HUVEC	ART	tumor growth↓	Chen et al., 2004a
Endothelial cells	HUVEC	DHA	VEGF-binding to its receptors↓, Flt-1↓, KDR/flk-1↓,neovascularization in chicken chorioallantoic membrane (CAM) assay↓	Chen et al., 2004b
		ART	neovascularization in CAM assay↓	Huan-Huan et al., 2004
Diverse	60 NCI cell lines	ARS, ART, ARE etc.	mRNA expression of iron-related genes correlated with IC_{50} values	Anfosso et al., 2006
Multiple myeloma	RPMI8226	DHA	VEGF secretion↓, neovascularization in CAM assay↓	Wu et al., 2006
Leukemia	K562	ART	VEGF secretion *in vitro* and *in vivo*↓	Zhou et al., 2007
Rat glioma	C6 (*in vivo*)	ARM	tumor growth↓, microvessel density↓	Wu et al., 2009
Multiple myeloma	RPMI8226	ART	VEGF and Ang-I secretion↓, neovascularization in CAM assay↓	Chen et al., 2010
Diverse	55 NCI cell lines	ART	mRNA expression of the angiogenesis promoting factor ITGB1 correlated with IC_{50} values	Sertel et al., 2010
Pancreatic carcinoma	BxPC-3-RFP	DHA	VEGF *in vivo*↓	Aung et al., 2011
Murine Lewis lung carcinoma	LLC	DHA	KDR/flk-1↓	Zhou et al., 2010
Endothelial cells, prostate carcinoma	HUVEC, BxPC-3	DHA	growth and tube formation↓, NF-κB binding↓, VEGF↓, IL8↓,COX2↓, MMP9↓, microvessel density *in vivo*↓	Wang et al., 2011
Pancreatic carcinoma	PC-3M		VEGF↓	Wang et al., 2012
Endothelial cells	HUVEC	DHA	VEGFR2↓,nuclear translocation of NF-κB↓, IκBα↑	Dong et al., 2014
Hepatocellular carcinoma	HepG3, BWTG3, Diethylnitrosamine-induced tumors	ART	VEGF *in vitro* and *in vivo*	Vandewynckel et al., 2014
Cervical carcinoma	ME-180	ARS	VEGF↓	Mondal and Chatterji, 2015
Endometrial cells	HUVEC	DHA	ERK1/2↓, ERK1/2 phosphorylation, FOS↓, MYC↓	Dong et al., 2015

INHIBITION OF SIGNALING PATHWAYS

There is also evidence that artemisinins influence tumor growth by inhibition of several signal transduction pathways [53,68,159,173,175,201-216] (**Table 9**). The Wnt/β-catenin pathway is affected by down-regulation of β-catenin, and translocation of β-catenin from the nucleus to the cell membrane. Artemisinins shut down EGFR signaling in epidermal tumor cells and BCR/ABL in leukemia cells. Furthermore, transcription factors such as mTOR, MYC/MYX, NF-κB, AP-1 (FOS/JUN), CREB and others are inhibited by ARS-type compounds.

Importantly, artemisinins inhibit cell invasion, migration and metastasis. Major metastatic regulators such as tubiquitous plasminogen activator (u-PA) and metalloproteinases (MMPs) were downregulated by ART. This drug inhibited the expression of MMP-2 and MMP-7 mRNA/protein in lung cancer cells. In luciferase reporter assays, ART downregulated MMP-2-, MMP-7- and u-PA-promoter/-enhancer activity, in parallel to AP-1- and NF-κB-transactivation[211]. In breast cancer cells, ART inhibited the transcription, expression and activity of MMP-1[60].

Another interesting target of artemisnins is the translationally controlled tumor protein (TCTP). It has first been reported in *Plasmodia* that ARS binds to this protein[217,218]. TCTP is linked to cellular growth control ubiquitously expressed in all eukaryotic organisms from protozoa such as *Plasmodium* to plants and mammals[219].

The interaction of *Plasmodium falciparum* TCTP (PfTCTP) with ARS was also reported[220]. The crystal structure of PfTCTP was determined by cloning and expression of the *PfTCTP* gene. Using mass spectrometry, bioinformatic approaches and surface plasmon resonance spectroscopy, novel binding sites of ARS were identified, which are in direct neighborhood to amino acids 19–46, 108–134 and 140–163. The regions covered by these residues are known to be functionally important for TCTP function.

As the name implies, TCTP also plays a role in tumor cells. TCTP is involved regulating cell cycle transition, apoptosis, calcium homeostasis, and cytoskeleton, and interestingly enough, in tumor reversion. This phenomenon is characterized by the inhibition or loss of key events that are necessary for tumor transformation. As a result, tumor cells revert to normal cells[221,222].

Recently, a novel approach to identify ARS-interacting target proteins in cancer cells was presented[223]. Our approach overcomes usual problems in traditional fishing procedures, because the drug was attached to a surface without further chemical modification. The proteins identified effect among others, cell cycle arrest, apoptosis, inhibition of angiogenesis, disruption of cell migration, and modulation of nuclear receptor responsiveness. Furthermore, a bioinformatic approach confirmed experimentally identified proteins and suggested a large number of other interacting proteins. Among the identified proteins was also TCTP.

Inhibition of TCTP by artemisinins opens the possibility that ARS-type drugs inhibit tumor growth not only by induction of apoptosis or other forms of cell death, but also by the induction of cellular differentiation and tumor reversion. Differentiation therapy represents an attractive treatment strategy, as it is not associated with the typical, severe side effects of clinically established cytotoxic chemotherapy. All-trans-retinoic acids are examples for the potential of differentiation or tumor reversion therapy for acute promyelocytic leukemia[224,225]. Whether artemisinins represent another class of drugs suitable for differentiation and tumor reversion therapy, deserves further investigation.

PERSPECTIVES: CLINICAL ACTIVITY IN CANCER PATIENTS

A plethora of results acquired during the past two decades shed light on the anticancer activity *in vitro* and *in vivo* and the molecular modes of action of artemisinins. Several research teams in Europe, Asia and America confirmed the inhibitory effects of artemisnins against tumors under experimental conditions. We feel that the time has come now to translate these promising data from the preclinics to the clinics. This is a specifically burning question, since we know from malaria treatment that artemisinins are well tolerable and that the toxicity of these compounds are rather modest and are much less than those known from standard anticancer drugs[226]. The entire toxicological assessment of ARS-type drugs that have been done in the context of development of these drugs as antimalarials may be used as basis for their investigation as anticancer drugs. This beneficial circumstance may speed up the further clinical development of artemisinins as anticancer drugs.

Veterinary tumors

In this context, it is of interest that cancer is not only a problem for human health, but that other mammals also spontaneously develop tumors. This is of practical relevance in veterinary medicine. Pets like dogs or cats suffering from tumors are treated with surgery, radiotherapy, or chemotherapy in a somehow comparable manner as human parients too. This circumstance offers the exciting opportunity to study the anticancer activity of artemisinins under clinical conditions in pets.

A safety/efficacy field study with ART was conducted in 23 dogs with non-resectable tumors[227]. ART was administered for 7-385 days at a dosage of 651-1178 (median 922) mg/m^2. No neurological or cardiac toxicity was observed and 7 dogs exhibited no adverse effects at all. Fever and haematological/gastrointestinal toxicity, mostly transient, occurred in 16 dogs. One dog died from pneumonia. Plasma ART and DHA levels fell below the limit of detection within 8-12 h after artesunate administration, while levels after two hours were close to 1 μM. Artesunate produced a long-lasting complete remission in one case of cancer and short-term stabilization of another seven cases.

Recently, the use of capsules containing powder of Herba Artemisiae annuae was reported to treat pet sarcoma[228]. The surgical tumor removal as standard treatment was supplemented by adjuvant therapy with *A. annua*. One cat and one dog with fibrosarcoma survived 40 and 37 months,

Table 9. Effect of artemisinins on signaling pathways in tumor cells.

Tumor type	Cell line	Drug	Effect	Reference
Wnt/β-catenin pathway:				
Colorectal carcinoma	HT-29	ART	Translocation of β-catenin from nucleus to adherent junctions of membrane, β-catenin-mediated transcription↓, hyperactive Wnt/β-catenin signaling pathway↓	Li et al., 2007
Colorectal carcinoma	HT-29	ART	Membraneous translocation of β-catenin, E-cadherin↑, reversion of EMT	Li et al., 2008
Mouse normal macrophages	RAW 264.7	ART	Involvement of cAMP-mediated and Wnt/β-catenin signaling pathways.	Konkimalla et al., 2008
Osteosarcoma		DHA	β-Catenin↓ because of increased catalytic activity of GSK3β, Wnt/β-catenin signaling↓	Liu et al., 2013
Receptor signaling:				
Diverse	55 NCI cell lines, transfected cells	ART	mRNA expression of EGFR and EGFR-downstream genes correlated with IC_{50} values. Cell lines transfected with EGFR downstream genes were more sensitive to ART than wild-type cells. Inhibition of the EGFR-RAS-Raf-MEK-ERK pathway.	Konkimalla et al., 2009
Leukemia	K562	DHA	BCR/ABL↓,downstream signal transducers (AKT and ERK1/2 tyrosine kinase activity↓,NF-κB protein expression↓	Lee et al., 2012; 2013
		ARS, ART, DHA etc.	A network pharmacology approach revealed five major pathways: PI3K/AKT, T cell receptor, Toll-like receptor, TGF-β and insulin signaling pathways	Huang et al., 2013
mTOR pathway:				
Neuroblastoma	SHSY5Y	ARS	AMP kinase signaling↑, mTOR/p70S6K/p S6 signaling↓	Tan et al., 2013
Rhabdomyosarcoma	Rh30, RD	DHA	mTOR signaling pathways↓	Odaka et al, 2014
Transcription factors:				
Diverse	60 NCI cell lines	ART	Promoter binding motif analyses of differentially expressed genes identified MYC/MAX as transcriptional regulators	Sertel et al., 2010
Macrophages	RAW 264.7	DHA	PMA-induced COX-2expression↓ and PGE2 production↓, PMA-induced NF-κBp65↓, C/EBPβ↓,c-JUN↓ and CREB nuclear translocation↓. PMA-induced phosphorylation of AKT↓ and MAP kinases (ERK, JNK, p38)↓	Kim et al., 2013
Metastatic signaling:				
Ovarian carcinoma	SKOV3, OVCAR3	DHA	FAK↓, MMP2↓, TIMP1↓, TIMP2↓	Tan et al., 2011
Fibrosarcoma	HT-1080	DHA	Cell invasion and migration↓, MMP-9↓, MMP-2↓. Inhibition of MMP-9 expression by NF-κB, inhibition of MMP-2 by MT1-MMP. No effect on TIMP-1 and TIMP-2	Hwang et al., 2010
Lung cancer (NSLCL)	H1395, A549, LXF289, H460, Calu3, H1299	ART	u-Pa↓, MMP-2↓, MMP-7↓, AP-1↓, NF-κB↓	Rasheed et al., 2010
Translationally Controlled Tumor Protein:				
		DHA	Binding to fortilin/TCTP, ubiquitination↓, proteasome-dependent shortening of TCTP half-life. TCTP-knock down cells were DHA-resistant, TCTP-transfectant cells were more DHA-sensitive	Fujita et al., 2008
Lung cancer	A549	DHA	*TCTP* mRNA↑, but TCTP protein↓. Increased TCTP protein secretion	Liu et al., 2014
Neurofibromatosis type 1 (NF1)	NF1-deficient Schwann cells, MPNST	ART	Binding and degradation of TCTP, MPNST↓ but not normal Schwann cells. TCTP level inversely correlated with ART sensitivity	Kobayashi et al., 2014
Other mechanisms:				
Mouse normal macrophages	RAW 264.7	ART	NO↓	Konkimalla et al., 2008
Hepatocellular carcinoma	HepG2	ART	NO↓, heme-harboring NOS↓	Zeng and Zhang, 2011
Pancreatic carcinoma	MiaPaCa-2, BxPC-3	ART	TOPO2A↓	Youns et al., 2009
	T-cells	DHA	Th cell differentiation↓, TGF-βR/Smad-dependent Treg generation↑,mTOR pathway↓.	Zhao et al., 2012

respectively, without tumor relapse. Two other dogs suffering from fibrosarcoma and hemangioendothelial sarcoma also showed complete remission and were still alive after 39 and 26 months, respectively. Fibrosarcoma and hemangioendothelial sarcoma are tumor types, which are primarily treated by surgical removal and survival times for dogs with fibrosarcoma treated with standard surgery are usually in a range from 7 to 12.2 months. Our results are remarkable, since the add-on therapy with *A. annua* capsules prolonged the survival times of the animals. *A. annua* was well tolerated without noticeable side effects. These four cases indicate that *A. annua* may be a promising herbal drug for cancer therapy. Interestingly, ARS is not the only cytotoxic compound in *A. annua,* and several other constituents are also cytotoxic towards cancer cells[229]. The plant extract may therefore be considered as "natural combination therapy", which might be even more beneficial for cancer therapy than treatment with isolated ARS alone.

Case reports of human cancer patients

Three patients treated with artemisinins responded well[230]. A 47-year-old female with breast cancer (stage 4) and metastases in her spine took ARS and showed tumor regression in computer tomography. Similar experiences were made in another breast cancer patient. A 47-year-old female suffering from terminal liver cancer and abdominal ascites took ARS and was still alive 2.5 years later[230]. Another case report has been published on the treatment of a laryngeal squamous cell carcinoma with ART[231].

Two patients with uveal melanoma were treated on a compassionate basis after standard chemotherapy was ineffective[232]. ART was well tolerated in both patients. One patient received fotemustine plus ART, which results in a temporary response, while the disease was progressing under prior fotemustine therapy alone. This patient died 23 months after entry in stage 4 disease. The second patient experienced a disease stabilization after application of dacarbazine and ART. Later on, the disease progressed with metastases in lung and spleen. This patient was alive at the time point of publication of this case report, which was 47 months after first diagnosis. The results of both treatment attempts with ART are remarkable in light that the median survival of uveal melanoma is two to five months.

Recently, longitudinal observations on the efficacy of *A. annua* in a prostate carcinoma patient were published[233]. The patient with prostate carcinoma (pT3bN1M1, Gleason score 8 (4+4)) staged by imaging techniques (MRT, scintigraphy, SPECT/CT) presented with a prostate specific antigen (PSA) blood level of >800 μg/L. After short-term treatment with bacalitumide (50 mg/d for 14 days) and long-term oral treatment with *A. annua* capsules (continuously 5 × 50 mg/d), the PSA level dropped down to 0.98 μg/L. MRT, scintigraphy and SPECT/CT verified tumor remission. Seven months later, blood PSA and ostase levels increased, indicating tumor recurrence and skeletal metastases. Substituting *A. annua* capsules by artesunate injections (2×150 mg twice weekly *i.v.*) did not prohibit tumor recurrence. PSA and ostase levels rose to 1245 μg/L and 434 U/L, respectively, and MRT revealed progressive skeletal metastases, indicating that the tumor acquired resistance. The high expression of MYC, TFR, and VEGFC in the patient biopsy as determined by immunohistochemistry corresponded with high expression of these markers in the ARS-sensitive PC-3 cells compared to ARS-resistant DU-145 cells. In conclusion, long-term treatment with *A. annua* capsules combined with short-term bicalitumide treatment resulted in considerable regression of advanced metastasized prostate carcinoma.

Clinical trials

Recently, ART and DHA pharmacokinetics have been characterized in patients with metastatic breast cancer during long-term (>3weeks) daily oral ART administration[234]. Twenty-three patients received ART orally (100, 150, or 200 mg OD). Pharmacokinetics of ART and DHA were well described by a combined drug metabolite model without any covariates and with an increase in apparent elimination clearance of DHA over time. The estimated DHA saliva/plasma ratio was in good agreement with the reported DHA unbound fraction in human plasma. Saliva ARS concentrations correlated poorly with plasma concentrations. This suggests the use of saliva sampling for therapeutic drug monitoring of DHA. Response to ART treatment or survival times were not recorded in this study.

As the binding affinity of artemisinin and its derivatives dihydroartemisinin and artesunate to blood serum proteins might influence the effectiveness of the drug, the binding of ARS and derivatives to serum albumin has been studied under near physiological conditions[235]. Binding kinetics indicate a simple, single-step association process for all ARS derivatives. The determined changes in enthalpy and entropy upon drug binding clearly indicate that hydrophobic forces are most important for ARS and DHA binding, whereas binding of ART is governed by both hydrophilic and hydrophobic forces. Key residues, which are most likely involved in binding of the respective compounds, were identified in subsequent protein/drug docking studies. The obtained results not only explain differences in between artemisinin and derivatives but generally illustrate how slight modifications in a drug can significantly affect principles underlying drug binding to target proteins. This result may be important for the performance of clinical trials with artemisinins for cancer therapy.

The efficacy and toxicity of the standard combination therapy of vinorelbine and cisplatin with or without ART artesunate has been compared in the treatment of advanced non-small cell lung cancer[236]. Each treatment group consisted of 60 patients. ART was applied as *i.v.* injection at a concentration of 120 mg from the 1st to the 8th day. At least two treatment cycles were performed. There were no significant differences in the short-term survival rate, mean survival time and 1-year survival rate between the trial group and the control group, The disease control rate of the trial group (88.2%) was significantly higher than that of the control group (72.7%) and the time to progression of the

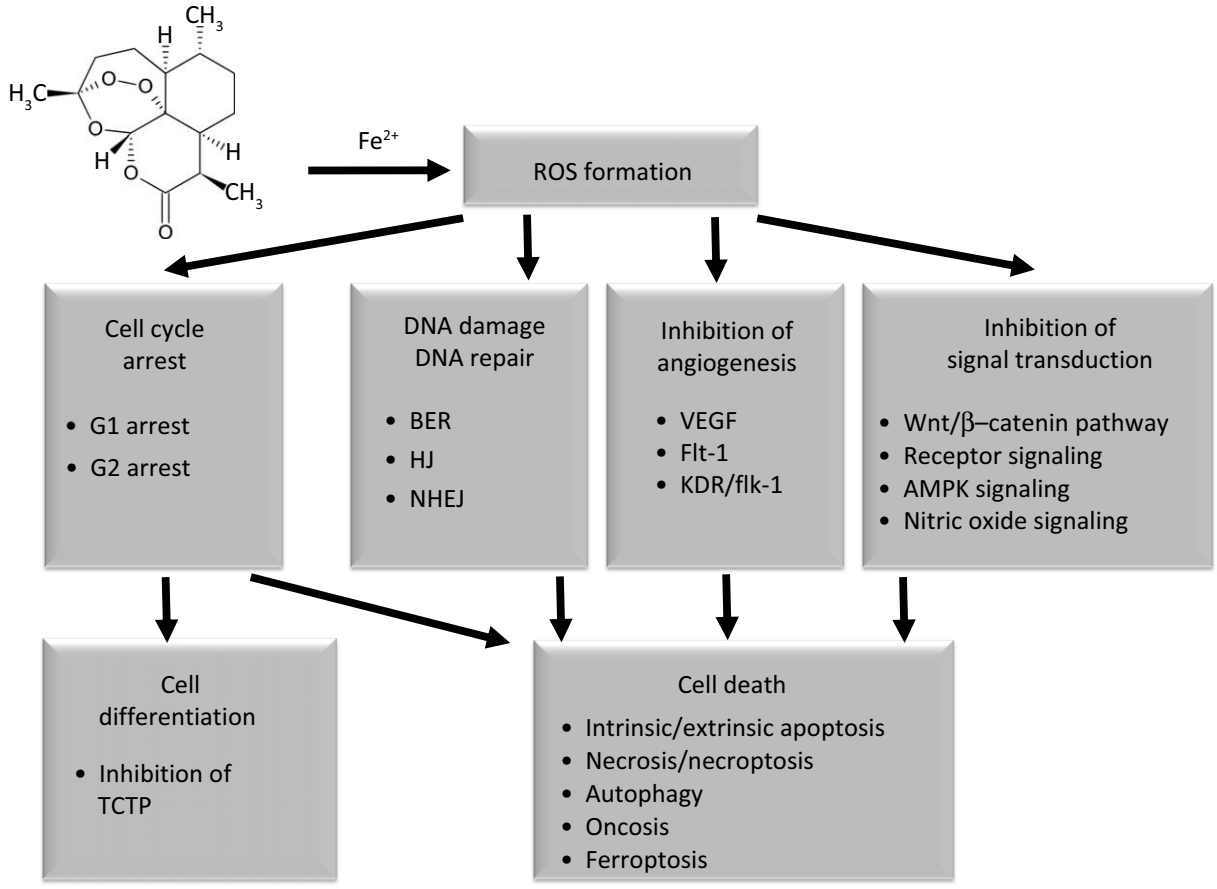

Figure 1. Synopsis of mechanisms of artemisinins in cancer cells.

ART-treated patients (24 weeks) was significantly longer than that of the control group (20 weeks). No significant difference was found in toxicity between these two groups. The authors concluded that ART in combination with standard chemotherapy elevated the short-term survival rate and prolonged the time to progression of patients with advanced non-small cell lung cancer without extra side effects.

Ten cervical carcinoma patients (stage III or IV) were treated with DHA for 28 days[237]. Clinical symptoms such as vaginal discharge and pain disappeared within three weeks in all patients with a median time of 7 days. Adverse events included headache and abdominal pain. No adverse events of grade 3 or 4 occurred. The immunohistochemical evaluation of tumor biopsies revealed that the expression of the tumor suppressor p53, the oncogene EGFR, and Ki-67 as nuclear proliferation marker, as well as the number of CD31-positivitely stained blood vessels stained decreased. On the other hand, the expression of transferrin receptor increased. Six patients experienced clinical relapse at an average of six months (range four to 8 months). Two patients died after 6-7 months remission. Four patients with relapse were treated a second time with DHA for 28 days, which resulted in clinical remission. Two of these patients subsequently died, 12-13 months after their first treatment cycle of DHA. Both of these patients died of renal insufficiency. The two other patients, who received the second treatment cycle as well as four patients, whose tumors did not relapse at the time point of publication of this study (median time of 9 months, range 2-24 months after first DHA treatment. The usual survival time prognosis of patients with metastasized cervical carcinoma at the Cancer Services, University Hospital (Treichville, Ivory Coast) is about four months. This prognosis compares to other hospitals in Africa, *e.g.* gynecological centers in Kigali, Rwanda and Nairobi, Kenya. It is remarkable that the median survival time of the four patients, who died during our study was 12 months (range 8 to 13 months). This phase I/II pilot study indicates on the clinical activity of DHA regarding improvement of the clinical symptoms and good tolerability of DHA in patients with advanced carcinoma of the cervix uteri.

A single center, randomized, double blind, placebo-controlled trial has been recently published on the use of ART in 23 colorectal carcinoma patients[238]. Patients received preoperatively either 14 daily doses of oral ART (200 mg; n = 12) or placebo (n = 11). The primary outcome measure was the proportion of tumor cells undergoing apoptosis (significant, if >7% showed Tunel staining). Secondary immunohistochemical outcomes assessed these tumor markers: VEGF, EGFR, c-MYC, CD31, Ki67 and p53, and clinical

responses. Twenty patients (ART = 9, placebo = 11) completed the trial per protocol. Randomization groups were comparable clinically and for tumor characteristics. Apoptosis in >7% of cells was seen in 67% and 55% of patients in ART and placebo groups, respectively. Using Bayesian analysis, the probabilities of ART treatment effect reducing Ki67 and increasing CD31 expression were 0.89 and 0.79, respectively. During a median follow up of 42 months, one patient in the ART and six patients in the placebo group developed recurrent tumors. It can be concluded that ART had anti-proliferative properties in colorectal carcinoma was generally well tolerated.

In conclusion, there is ample evidence for the activity of artemisinin and its derivatives against tumors. Artemisinin-type drugs exert multi-factorial cellular and molecular actions in cancer cells (**Figure 1**). Ferrous-iron mediated ROS formation contribute to the anticancer effects of artemisinins. Artemisinin-type drugs exert their cytotoxicity towards cancer cells by multiple mechanisms, which is a quite typical feature for many natural products. Artemisinins bear the potential to be used for veterinary and human cancer patients. Therefore, the clinical activity warrants further investigation in larger scale Phase II and III clinical trials.

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