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Review

From ancient herb to modern drug: *Artemisia annua* and artemisinin for cancer therapy

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ABSTRACT

Artemisia annua L. is used throughout Asia and Africa as tea and press juice to treat malaria and related symptomes (fever, chills). Its active ingredient, artemisinin (ARS), has been developed as antimalarial drug and is used worldwide. Interestingly, the bioactivity is not restricted to malaria treatment. We and others found that ARS-type drugs also reveal anticancer *in vitro* and *in vivo*. In this review, we give a systematic overview of the literature published over the past two decades until the end of 2016. Like other natural products, ARS acts in a multi-specific manner against tumors. The cellular response of ARS and its derivatives (dihydroartemisinin, artesunate, artemether, arteether) towards cancer cells include oxidative stress response by reactive oxygen species and nitric oxide, DNA damage and repair (base excision repair, homologous recombination, non-homologous end-joining), various cell death modes (apoptosis, autophagy, ferroptosis, necrosis, necroptosis, oncosis), inhibition of angiogenesis and tumor-related signal transduction pathways (*e.g.* Wnt/β-catenin pathway, AMPK pathway, metastatic pathways, and others) and signal transducers (NF-κB, MYC/MAX, AP-1, CREBP, mTOR *etc*). ARS-type drugs are at the stairways to the clinics. Several published case reports and pilot phase I/II trials indicate clinical anticancer activity of these compounds. Because of unexpected cases of hepatotoxicity, combinations of ARS-type drugs with complementary and alternative medicines are not recommended, until controlled clinical trials will prove the safety of non-approved combination treatments.

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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/EBPβ, CCAAT/enhancer binding protein beta; CAM, chorioallantoic membrane; CD, cluster of differentiation; 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 beta; 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 beta; 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 7protein 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-β, tumor growth factor beta; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor α; TOPO2A, 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.

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Contents

1. Introduction

Artemisinin is a 1,2,-trioxane from the Chinese medicinal plant Sweet Wormwood (*Artemisia annua* L., Asteraceae). The plant was first mentioned by Hong Ge (葛洪, (281–340 B.C.) as remedy to treat fever and chills in the "Handbook of Prescriptions for Emergency Treatment" (*Hou Bei Ji Fang*, 肘后备急方). The fact that it was still listed in the "Compedium of Materia Medica" (*Ben Cao Gang Mu*, 本草纲目), by Li Shizen (李时珍) in the year 1596 and is even known nowadays may be taken as a clue for its usefulness and activity.

In 1967, China's former chairman Mao Zedong initiated a research project to search for malaria-active medicinal plants from Chinese medicine. During the Vietnam War, the Vietnamese government asked China for help, because numerous Vietnamese soldiers suffered from malaria. Among the 500 scholars, who screened traditional Chinese plants and remedies, was Tu Youyou. She observed that *A. annua* was among the most active herbs. However, her results were not always repeatable with sufficient reliability. Going back to the ancient textbooks, Tu Youyou recognized that the recommended preparation of *A. annua* was not a hot decoction, as most frequently used standard procedure for medicinal herbs. Rather, the historical text described the use of a pressed juice of *A. annua*. Taking these details seriously, Tu Youyou then found that *A. annua* was more effective against *Plasmodia* infections, if she prepared low temperature extractions of this plant [1–3]. Bioactivity-guided fractionation subsequently allowed structure elucidation of sesquiterpene lactones of the artemisinin-type [2,4]. Together with its derivatives, artemisinin reached worldwide attraction, and artemisinin-based combination therapies nowadays belong to the established standard treatments of malaria worldwide [5–11]. As appreciation that artemisinin (ARS) helped to save millions of lifes, Tu Youyou was honored with numerous awards, including the Lasker DeBakey Clinical Research Award in 2011 and the Nobel Prize for Medicine or Physiology 2015 [12–15].

2. The antimalarial activity of artemisinin

In erythrocytes, *Plasmodium* trophozoites and schizonts feed on hemoglobin as the source for amino acids. Hemoglobin is toxic for *Plasmodia*, since heme-iron generates reactive oxygen species (ROS). Therefore, the malaria parasites convert hemoglobin to the non-toxic hemozoin [16,17]. During this reaction, the released heme-iron cleaves the endoperoxide bridge of ARS by a Fe(II) Fenton-type reaction, and free radical intermediates kill the *Plasmodia* [18–20]. Other mechanisms of the antimalarial activity of ARS include

- the inhibition of redox cycling,
- the inhibition of a glutathione S-transferase termed *Plasmodium falciparum* exported protein 1 (EXP1),
- the inhibition of *Plasmodium falciparum* PfATP6, which represents a sarcoendoplasmatic reticulum Ca^{2+} ATPase (SERCA),
- the inhibition of digestive vacuole cysteine protease, as well as
- the alkylation of specific parasite proteins, including translationally controlled tumor protein (TCTP) [21–26]

DNA lesions have not been observed in *Plasmodia*, in contrast to cancer cells [27].

3. Beyond malaria: activity of artemisinin to other diseases

Interestingly, numerous hints were accumulated during the past years, that activity of ARS is not restricted to malaria and that it may also be of therapeutic interest for several other diseases (Fig. 1). It was Tu Youyou, who provided first data that dihydroartemisinin may be beneficial for the treatment of Lupus erythematosus-related nephritis by inhibiting the production of anti-ds-DNA antibodies, the secretion of TNF-α, and NF-κB signaling pathway [28]. ARS-type drugs also revealed bioactivity towards viruses (*e.g.* human cytomegalovirus, HCMV), schistosomiasis, trypanosomiasis, cancer *in vitro* and *in vivo*, and even against plant tumors [29–37]. Resent results indicated that *A. annua* and ARS may not only be active against infectious and malignant diseases, but also to reduce glucose and act against diabetes mellitus [38,39].

Here, we present a timely review on the anti-cancer activity *in vitro* and *in vivo* of ARS and its derivatives artesunate (ART), artemether (ARM), arteether (ARE), as well as the first metabolite, dihydroartemisinin (DHA). Furthermore, we report on clinical data in cancer patients. Non-approved second generation derivatives, nanotherapeutic strategies with artemisinin-type compounds, as well as combination therapies involving ARS-type drugs were not included in this review. We searched the PubMed and Google Scholar databases with the following search term combinations: '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) 'angiogene-

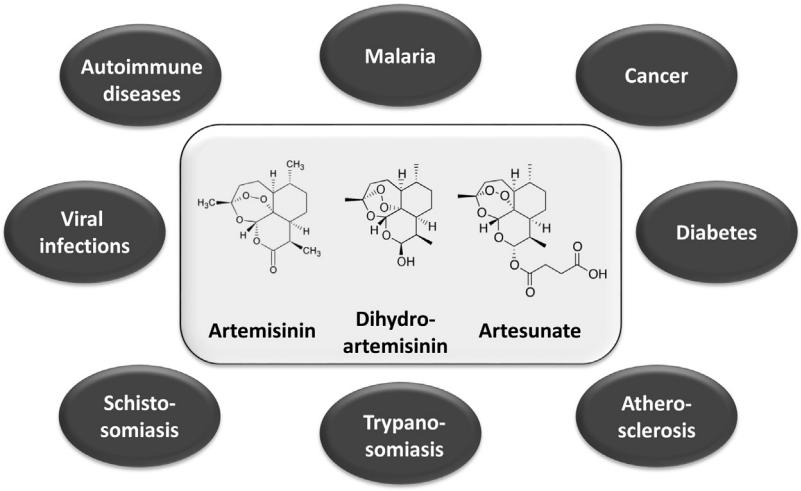

Fig. 1. Expanding the bioactivity of artemisinin and its derivatives.

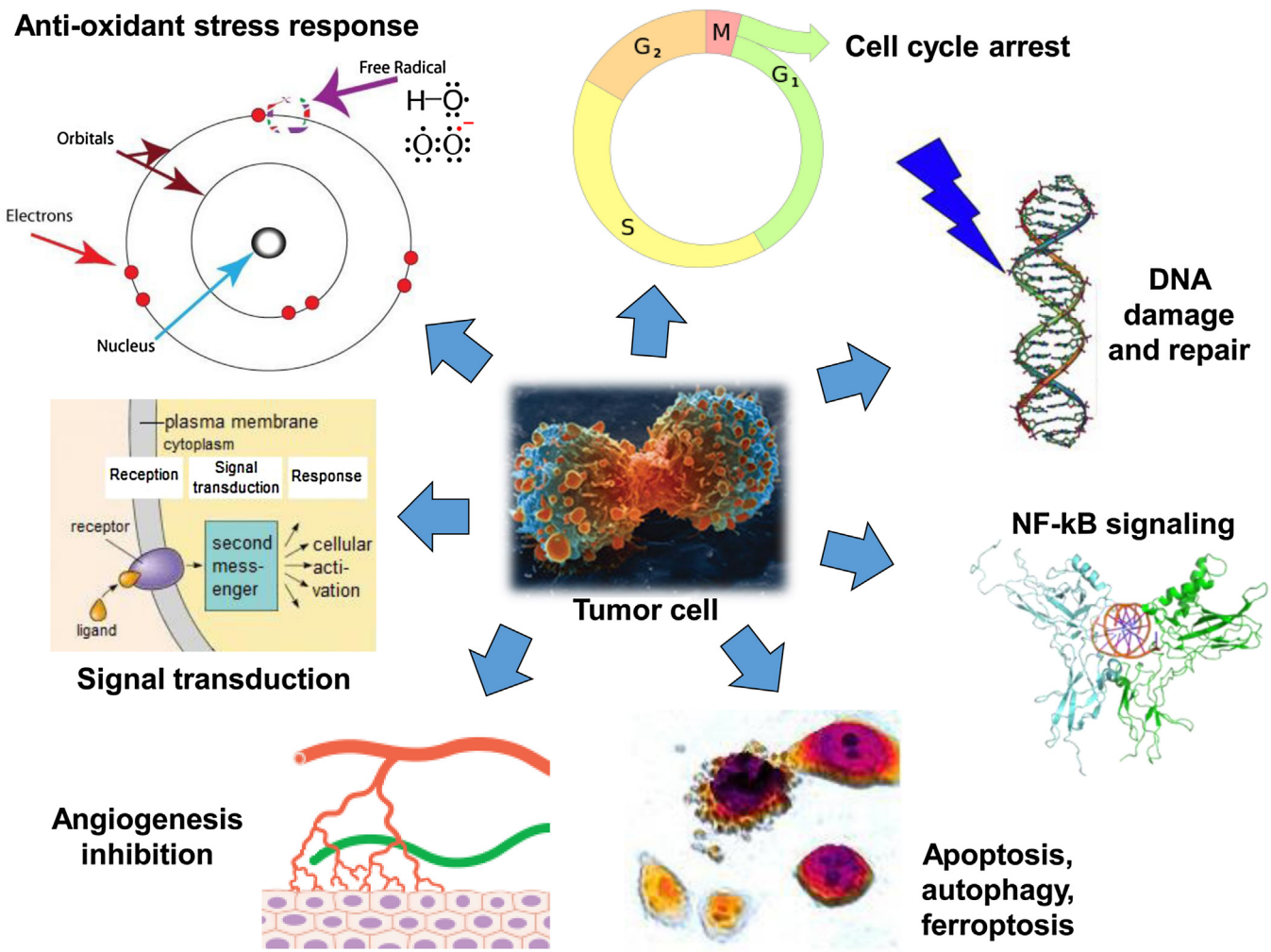

Fig. 2. Synopsis of mechanisms of action of artemisinin and its derivatives against tumor cells (all images were taken from Wikipedia).

sis/angiogenic', and (7) 'signaling/signal transduction'. The review encompasses the relevant literature until December 2016. In the first parts of the review, we report on the multiple cellular and molecular mechanisms that account for the activity of artemisinin-type drugs against tumor cells (Fig. 2). At the end, we summarize the preliminary evidence that artemisinin and its derivatives may exert clinial activity against tumors in patients.

4. Tumor inhibition in cell culture and animals

In the 1990s, both Chinese and Western groups (including my own) described the cytotoxicity of ARS and related compounds (DHA, ART, ARM, ARE) towards tumor cell lines [33–36]. In subsequent years, numerous papers confirmed these initial results, and there is now mounting evidence that ARS-type compounds act

against tumor cells *in vitro*. The endoperoxide moiety is essential for bioactivity, since ARS-like compounds without it were inactive [36,37].

The profound activity towards tumor cell *in vitro* (including stem-like cancer cells) [40–45] was confirmed by *in vivo* investigations [40–44]. Numerous experiments showed the inhibition of transplantable tumors in mice (Table 1, [46–80]). Most animal experiments have been performed by using human xenograft tumors transplanted to nude mice. This represents a frequently used standard animal model in cancer research, because human tumor cells can be investigated *in vivo* outside the human organism in animals. A disadvantage is that athymic mice lack an intact immune system. Therefore, some researchers investigated syngeneic models, *i.e.* rodent tumors transplanted to mice or rats. The activity of ARS-type compounds against both xenograft or syngeneic tumor models provided compelling evidence for the anticancer activity of this drug class in animals. Remarkably, the antitumor effects were not only shown in subcutaneously transplanted tumors, but also in orthotopically transplanted ones, which resembles much better tumor growth in human patients.

5. Oxidative stress response

The endoperoxide moiety is crucial for the bioactivity of artemisinin-type drugs. Its cleavage leads to ROS formation and presumably oxidative stress. To get deeper insight in the role of oxidative stress, we correlated the $\log_{10}IC_{50}$ values of 55 tumor cell lines for ART with their microarray-based transcriptome-wide expression in collaboration with the National Cancer Institute (NCI, USA). Interestingly, numerous statistically significant associations were found between cellular response to ART and mRNA expression of genes involved in oxidative stress 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*) [81–84]. We used cell lines transfected with cDNAs for some of these antioxidant genes (*CAT*, *SOD1, TXN,* or *GLCLR*) to verify the microarray-based results. Indeed, the transfected cells were more resistant to ART as their non-transfected or mock vector-transfected counterparts [92,93]. Furthermore, inhibitors for γ-glutamylcysteine synthetase (*i.e.* buthionine sulfoximine, BSO) or glutathione S-transferases (*i.e.* ethacrynic acid, EA) sensitized cells to ART, prodiving another clue for the causative role of oxidative stress response for cellular responsiveness of tumor cells towards ART [84].

In subsequent years, a plethora of data published by other authors confirmed our initial results on oxidative stress by ARS-type drugs. ROS generation by ARS, ART or DHA has been published in epithelial, mesenchymal, or hematopoietic cell lines derived from many different tumor origins (Table 2, [52,67,71,73,75,77,83–102]). Supportive evidence for the causative role of ROS came from experiments using prooxidant substances (vitamins C and D3, dexamethasone), which increased ARS-mediated cytotoxicity and by antioxidants and ROS scavengers (N-acetyl-cysteine (NAC), vitamin E) reducing ARS-conferred tumor cell death (Table 2).

6. Role of iron for artemisinin's activity

There is a plethora of publications indicating a crucial role of iron for the antimalarial, but also for the anticancer activity of ARS-type drugs. Ferrous sulfate and holotransferrin enhanced DHA's activity against rat fibrosarcoma and breast carcinoma [103,104]. Iron(II)–glycine sulfate (Ferrosanol®) and holotransferrin increased the cytotoxicity of maltosyl-β-cyclodextrin-encapsuled ARS, ART, and ART against leukemia and astrocytoma cells [105]. The enhancement of ART cytotoxicity by ferrous iron or transferrin was reversed by a monoclonal anti-transferrin receptor antibody, which competes with transferrin for receptor binding. This is another hint for the specificity of iron-mediated cellular effects. We found that tumor cells expressed significantly more transferrin receptor on their cell surface than normal cells, which indicates that ferrous iron and transferrin, respectively, might at least in part boost ART cytotoxicity in a tumor-specific manner. Furthermore, Ferrosanol® enhanced ART-induced cytotoxicity in most, but not all of tumor cell lines of the Oncotest panel [106]. The extent of enhanced cytotoxicity of ART by Ferrosanol® significantly correlated with the transferrin receptor expression in the cell lines with high transferrin receptor expression. This result was confirmed in the NCI panel of tumor cell lines. Here, cellular response to ART did not only correlate with transferrin receptor mRNA expression [106].

Transferrin receptor is not the only protein involved in cellular iron homeostasis. We investigated the ATP-binding cassette (ABC) transporter, ABCB6 [106]. In the NCI cell line panel, there also was a significant association between the $\log_{10}IC_{50}$ values for ART and *ABCB6* mRNA expression. ART treatment of leukemia or breast cancer cells induced *ABCB6* expression. Furthermore, antisense oligonucleotides directed against *ABCB6* inhibited the growth-reducing and differentiation-inducing effects of ART.

In conclusion, our results indicated that increased cytotoxic effects of ART by the addition of ferrous iron or transferrin may not always be observed. Since iron can act as cofactor for enzymes involved in cellular proliferation, unwanted, negative effects cannot be excluded, which rather promote than inhibit tumor growth [107]. Therefore, a clinical coapplication of ART plus ferrous iron or transferrin is rather not recommendable.

These initial results on the enhancing effect of ferrous iron for the cytotoxicity of ARS-type drugs data in tumor cells have been also found by numerous authors in subsequent years (Table 3, [48,53,92,103–106,108–126]). A strong hint for the role of iron came from the use of the iron chelator deferroxamine, which was able to diminish the anticancer effects of DHA. Recently, a novel from of iron-dependent cell death termed ferroptosis has been described [127], and ART has been observed to be tightly linked with characteristics of ferroptosis [124,128].

It is well-known that cancer cells contain more iron than normal tissues [129,130] explaining at least partially that ARS-type drugs preferentially kill cancer cells than nomal cells. Transferrin-iron complexes are internalized by the transferrin receptor. Its expression is very high in tumors [129–134], but low in normal tissues and restricted to few sites, *e.g.* basal epidermis, endocrine pancreas, hepatocytes, Kupffer cells, testis, and pituitary [131]. Therefore, it is worth investigation the question, whether or not transferrin receptor and other iron-regulating proteins may serve as biomarkers to predict the responsiveness of tumors to ARS-type drugs.

7. Induction of DNA lesions

Previously, we found statistically significant relationships between the $\log_{10}IC_{50}$ values of various ARS-type drugs and the microarray-based mRNA expression of several DNA damage response and repair genes in the NCI panel of tumor cell lines, *e.g. ERCC5, FEN1, HMG1, HMF17, LIG1, RPS3, UNG,* and *UBE2A* [82,83]. We speculated whether ARS and its derivatives induce DNA lesions owing to the cleavage of the endoperoxide moiety, which causes ROS- or carbon-centered radical-mediated DNA damage.

Table 1
Anticancer activity *in vivo* of ARS-type drugs.

Cell line	Model type	Drug	Effect	Reference
Hematopoietic origin:				
U937 leukemia	Xenograft	DHA	Tumor growth↓, induction of apoptosis, ERK↓	Gao et al. [57]
DBA2/P815 murine mastocytoma	Syngeneic	ARS	Tumor growth↓	Tilaoui et al. [65]
Acute myeloic leukemia	Xenograft	ART, DHA	Tumor growth↓	Drenberg et al. [69]
Mesenchymal origin:				
KS-IMM Kaposi sarcoma	Xenograft	ART	Tumor growth↓, vacularization of matrigel plugs↓	Dell'Eva et al. [47]
Osteosarcoma		DHA	Tumor growth↓, β-catenin↓, GSK3β↑	Liu et al. [62]
Epithelial origin:				
HepG3 and Hep3 B hepatoma	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. [49]
Hepatocellular carcinoma	Xenograft	DHA	Tumor growth↓	Zhang et al. [61]
HepG2 and BWTG3 hepatocellular Ca	Xenograft		Tumor growth↓, microvessel density↓	Vandewynckel et al. [66]
Liver Ca	Syngeneic	ART	Prevention of hepatocarcinogenesis	Ilamathi et al. [70]
HO-8910 ovarian Ca	Xenograft	ART	Tumor growth↓, VEGF↓, KDR/flk-1↓	Chen et al. [46]
HO8910PM ovarian Ca	Xenograft, orthotopic	DHA	Tumor growth↓, metastasis↓, CD31↓, pFAK↓, MMP2↓	Wu et al. [60]
Ovarian Ca	Xenograft	ART	Tumor growth↓	Greenshields et al. [77]
Colorectal Ca	Xenograft	ART	Tumor growth↓, liver metastasis↓, Wnt/β-catenin pathway↓	Li et al. [49]
BxPC-3 pancreas Ca	Xenograft	DHA	Tumor growth↓, PCNA↓, cyclin D1↓, WAF1/C1P1↑, Bax↑, Bcl-2↓ caspase-9↑,	Chen et al. [51]
Pancreas Ca	Xenograft	ART	Tumor growth↓	Du et al. [52]
Pancreas Ca	Xenograft	DHA	Tumor growth↓, involvement of 4 microRNAs	Li et al. [74]
MTLn3 breast Ca	Syngeneic	ARS-TF	Tumor growth↓	Lai et al. [53]
MDA-MB-231 breast Ca	Xenograft	ART	Minimal inhibition due to resistance, NF-κB↑	Bachmeier et al. [55]
Breast Ca	Syngeneic	ART	Tumor growth↓, depletion of splenic CD4*, CD25+, Foxp3+ and Treg cells IL4↑, IFN-gamma↑	Farsam et al. [56]
MCF7 breast Ca	Xenograft	ARS	Tumor growth↓	Tin et al. [59]
MDA-MB-231 breast Ca	Xenograft	DHA	Inhibition of breast cancer- induced osteolysis	Feng et al. [80]
C6 rat glioma	Syngeneic, orthotopic		Tumor growth↓, microvessel density↓	Wu et al. [54]
T269 and LN-Z308 glioblastoma	Xenograft	DHA	Tumor growth↓	Lemke et al. [73]
A549 lung Ca	Xenograft	ART	Tumor growth↓, induction of apoptosis, EGFR↓, AKT↓, ABCG2↓	Ma et al. [256]
A549 and H1299 lung Ca	Xenograft, syngeneic	ART, DHA	Tumor growth↓	Tong et al. [76]
SGC 7901 gastric Ca	Xenograft	DHA	Tumor growth↓, metastasis↓	Sun et al. [63]
BGC-823 gastric Ca	Xenograft	ART	Tumor growth↓	Zhou et al. [64]
BGC-823 gastric Ca	Xenograft	DHA	Tumor growth↓	Zhang et al. [78]
HeLa and Hela/DHA cervix Ca	Xenograft	DHA	Inhibition of tumor growth more in sensitive HeLa than in DHA-resistant HeLa/DHA, overexpression of DJ-1 (PARK7)	Zhu et al. [67]
Rat gallbladder Ca	Syngeneic, orthotopic		Tumor growth↓	Zuo et al. [68]
Eca109 and Ex9706 esophageal Ca	Xenograft	ART	Tumor growth↓	Liu et al. [79]
Gallbladder Ca	Xenograft	ARS	Tumor growth↓	Jia et al. [71]
Head and neck Ca	Xenograft	DHA	Tumor growth↓	Jia et al. [72]
Head and neck Ca	Xenograft	ART	Tumor growth↓	Roh et al. [75]

By using the comet assay (single-cell gel electrophoresis), we identified indeed a dose-dependent induction of DNA strand breaks by ART [135]. This result was corroborated by detecting the expression of γ-H2AX. This is a DNA-related histone, whose expression is induced upon DNA double-strand breaks (DSB). We found a dose-dependent increase of γ-H2AX [135]. POLB-deficient cells were less resistant to ART than their wild-type counterparts. This result can be taken as a hint that ART-induced oxidative DNA lesions are repaired by the base excision repair pathway. Cells defective in homologous recombination (HR) due to inactivation of XRCC2 and BRCA2, respectively, as well as cells defective in non-homologous end-joining (NHEJ) due to inactivation of Ku80 were also less resistant to ART than wild-type cells. It can be concluded that ART-induced DSBs were repaired by the HR and NHEJ pathways [135].

ART forms oxidative DNA lesions, leading to formamidopyrimidine DNA glycosylase-sensitive sites and the generation of 8-oxoguanine and 1,N^6-ethenoadenine. Knockdown of Rad51 by siRNA and inactivation of DNA-PK considerably reduced resistance of tumor cells to ART, which also points to the role of HJ and NHEJ pathways for ART-induced DNA strand break repair. The cellular response to ART-induced DNA lesions included phosphorylation of several DNA-damage response signal transducers, such as ATM, ATR, Chk1, and Chk2 [136]. These results with ART were corroborated by other authors for other ARS-type drugs (Table 4, [81,82,135–141]).

8. Cell cycle arrest

In general, ROS and oxidative DNA lesions tremendously affect cellular integrity, leading to perturbations in cellular replication and division mechanisms, which ultimately cause cell cycle arrest and cell death. This is also true for ART-type drugs (Table 5, [50,51,59,61,63,71,76,77,79,82,97,115,120,123,142–161]). Cell cycle arrest has been reported to occur at G1 or G2 checkpoints, presumably depending on individual defects of tumor cell lines in the cell cycle machinery.

We treated 7 cell lines with ART under comparable conditions (regarding the same cultivation conditions, the same detection method, the same experimentator *etc.*). Three of them arrested the cell cycle at the G1 phase and the others at the G2 phase [147]. This was not only conceivable with the published data of other authors, but also indicated that the heterogeneous results in the lit-

Table 2
Oxidative stress induced by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
Thymoma	ART	Transfection of antioxidant genes (thioredoxin, manganese superoxide dismutase, catalase) induced resistance to ART	Efferth et al. [87]
Leukemia	ART	Correlation of 12 glutathione-related genes with IC_{50} values.	Efferth and Volm [84]
Jurkat, CCRF-CEM, CEM/ADR5000 leukemia	ART	ROS↑, ROS scavenging by NAC conferred ART resistance	Efferth et al. [86]
K562 leukemia	DHA	ROS↑	Wang et al. [92]
Molt-4 leukemia	DHA	Prooxidants increased cell death (vitamin C, vitamin D3, dexamethasone, H_2O_2). Antioxidants decreased cell death (vitamin E)	Gerhardt et al. [101]
Ramos non-Hodgkin lymphoma	ART	ROS↑	Sieber et al. [87]
Multiple myeloma	ART	ROS↑	Papanikolaou et al. [99]
Mesenchymal origin:			
ERMS embryonal rhabdomyosarcoma	ART	ROS↑, ROS-dependent expression of miR-133a and miR-206	Beccafico et al. [100]
Epidermal origin:			
Panc-1, BxPC-3, CFPAC-1 pancreas Ca	ART	ROS↑	Du et al. [52]
BxPC-3, PANC-1 pancreas Ca	DHA	ROS↑, ROS-mediated upregulation of death receptor DR5	Kong et al. [91]
Pancreas Ca	DHA	ROS↑	Jia et al. [96]
RIN pancreas Ca	ARS	ROS↑	Noori et al. [98]
PDAC pancreas Ca	ART	ROS↑	Eling et al. [102]
ASTC-a-1 lung Ca	DHA	ROS↑	Lu et al. [88]
ASTC-a-1 and A549 lung Ca	ART	ROS↑	Zhou et al. [93]
A549 lung Ca		ROS↑	Gao et al. [94]
A549 lung Ca	ART	ROS↑, ROS scavenging by NAC conferred ART resistance	Ganguli et al. [95]
HepG2 liver Ca	DHA	ROS↑	Gao et al. [89]
A375 melanoma	DHA	Oxidative and genotoxic stress response genes ↑	Cabello et al. [90]
HCT-116 colorectal Ca	DHA	ROS↑	Lu et al. [97]
HeLa and HeLa/DHA cervix Ca	DHA	DJ-1 conferred DHA resistance by ROS removal	Zhu et al. [67]
Gallbladder Ca	ARS	ROS↑	Jia et al. [71]
Head and neck Ca	ART	ROS↑, GSH↓, activation of Nrf2 pathway	Roh et al. [75]
Ovarian Ca	ARS	ROS↑	Greenshields et al. [77]
T269 and LN-Z308 glioblastoma	DHA	ROS↑, ROS scavenging by NAC conferred DHA resistance	Lemke et al. [73]
Diverse origin:			
NCI cell lines	ART	Correlation of microarray-based antioxidant gene expression with IC_{50} values.	Efferth and Oesch [83]
NCI cell lines	ART	Correlation of 12 glutathione-related genes with IC_{50} values.	Efferth and Volm [84]

erature may not be due to heterogeneous experimental conditions (Table 5).

The tumor suppressor p53 represents an important regulator of the cell cycle and of G1 arrest. We investigated p53 and $p21^{WAF1/CIP1}$ in wild-type HCT-116 colon cancer cells ($p53^{+/+}$ and $p21^{WAF1/CIP1+/+}$) and two isogenic knockout lines ($p53^{-/-}$, $p21^{WAF1/CIP1-/-}$ and $p53^{-/-}/p21^{WAF1/CIP1-/-}$) [82]. As measured by the incorporation rate of bromodeoxyuridine (BrdU), the two knockout cell lines were similarly sensitive to ART as the wild-type cells. The p53 protein expression was induced in wild-type but not knockout cells. ART strongly up-regulated the expression of $p21^{WAF1/CIP1}$ protein in wild-type cells, but only weakly in $p53/p21^{WAF1/CIP1}$ knockout cells. G1 arrest induced by ART was associated with down-regulation of both RB phosphorylation and CDK2 kinase activity. Upon ART treatment, protein expression and kinase activity of the G2/M regulator cyclin B1 was reduced in all three cell lines [90]. In a cell model transfected with a tetracycline repressor expression vector, conditional *CDC25A* gene expression enhanced the sensitivity towards ART, which also supports the view that ART induces G1 arrest [82].

Other cell lines inducing G2 arrest upon ART treatment showed signs of defective cytokineses, such as multiple centrosomes, small multiple spindles and multi-nucleated cells. Therefore, we specifically deleted the mitotic spindle checkpoint genes *bub1, bub2, bub3, mad1, mad2* and *mad3* and measured growth of wild-type and mutant cells after ART treatment. The *Δbub3* and *Δmad3* mutants showed were more sensitive and the Δmad2 mutant slightly less senstive than wild type cells. As Bub3, Mad3 and Mad2 proteins belong to the mitotic spindle checkpoint regulators, ART may interfere with this control mechanism [137,147].

As depicted in Table 5, other authors also found that ARS-type drugs induced G1 arrest by altering the expression of G1-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. G2 arrest induced by ARS-type drugs was accompanied by down-regulation of cyclin B and CDC25 B and up-regulation of cyclin D1. These results indicated that G1 or G2 cell cycle arrest was differently induced in individual tumor cell lines depending on specific cell cycle blockage mechanisms.

As shown by Hargraves and colleagues [162], cell cycle arrest was regulated by microRNAs. ARS and ART dose-dependently increased miR-34a expression leading to down-regulation of CDK4 expression and G0G1 arrest of breast cancer cells. The increased miR-34a expression induced by ARS or ART occurred independent of the mutational status of the tumor suppressor 53 in the cell lines investigated. Transfection of miR-34a inhibitors abolished the ARS/ART-mediated CDK4 downregulation and cell cycle arrest.

Table 3
Iron-mediated enhancement of cytotoxicity of ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
Molt-4 leukemia	DHA	Holotransferrin increased DHA cytotoxicity	Singh and Lai [108]
Molt-4 leukemia	ARS-TF	Transferrin tagging increased ARS cytotoxicity	Lai et al. [109,110]
CCRF-CEM leukemia	ARS ART	Iron(II)-glycine sulfate (Ferrosanol®) and holotransferrin enhanced the cytotoxicity of artemisinins, while the monoclonal anti-transferrin receptor antibody RS10 decreased it.	Efferth et al. [105]
K562 leukemia	DHA	Iron-loaded cells underwent autophagy and downregulation of transferrin-receptor expression	Wang et al. [92]
Molt-4 leukemia	DHA	Deferoxamine attenuated DHA cytotoxicity	Chan et al. [119]
Jurkat leukemia	DHA	Holotransferrin increased DHA cytotoxicity	Wang et al. [125]
Mesenchymal origin:			
Rat fibrosarcoma	DHA	Ferrous sulfate retarded tumor growth following DHA	Moore et al. [103]
DH82 canine histiocytic sarcoma	DHA	Erythrophagocytic uptake of heme-iron enhanced DHA cytotoxicity, suggesting a role of exogenous heme	Chikazawa et al. [121]
Breast carcinoma	DHA	Holotransferrin increased DHA cytoxicity	Singh and Lai [104]
U373 astrocytoma	ARS ART	Iron(II)-glycine sulfate (Ferrosanol®) and holotransferrin enhanced the cytotoxicity of artemisinins, while the monoclonal anti-transferrin receptor antibody RS10 decreased it.	Efferth et al. [105]
Epithelial origin:			
C6 rat glioma	ARS, DHA	Ferrous ions increased, deferoxamine abolished cytotoxicity.	Lu et al. [111]
C6 rat glioma	DHA	Transferrin enhanced DHA cytotoxicity	Kim et al. [126]
MTLn3 rat breast Ca	ARS-TF	Inhibition of tumor growth, no side effects	Lai et al. [53]
MCF-7 breast Ca	ART	Iron induced mitochondrial apoptosis, deferoxamine abolished cytotoxicity.	Hamacher-Brady et al. [114]
HCT-116 colorectal Ca	DHA	Iron-dependent endplasmic reticulum stress. GRP78↑, GADD153↑, deferoxamine aboslished these effects	Lu et al. [115]
HeLa cervix Ca	ARS	Heme and holotransferrin enhanced endoperoxide activation and cytotoxicity.	Mercer et al. [116]
	DHA	DHA depleted cellular iron and down-regulated transferrin receptor expression by a lipid raft-mediated internalization pathway	Ba et al. [117]
DU145 prostate Ca	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. [112]
HepG2, SK-HEP1 and LS174T liver Ca	ARS and others	Ferrosanol®, but not hemin increased cytotoxicity	Blazquez et al. [118]
HepG2 liver Ca	ARS, ART, DHA	Binding to transferrin enhanced cellular uptake	Yang et al. [122]
RB-Y79 retinoblastoma	ART	ART internalization was dependent upon transferrin receptor expression, siRNA-mediated knockdown of transferrin receptor decreased ART	Zhao et al. [120]
A549 lung Ca	ARS, ART, DHA	Binding to transferrin enhanced cellular uptake	Yang et al. [122]
Caki-1, 786-0 and SN12C-GFP-SRLu2 kidney Ca	ART	Transferrin receptor expression was correlated with metastasis and unfavorable prognosis. ART cytotoxicity correlated with transferrin receptor expression	Jeong et al. [257]
Diverse origin:			
36 Oncotest 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. [106]
Diverse	ARS	Heme (Fe^{2+} protoporphyrin IX) increased ARS cytotoxicity	Zhang and Gerhard [113]
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. [124]

9. Modes of programmed cell death

9.1. Apoptosis

Oxidative stress and DNA damage not only block cell cycle progression, but also induce apoptosis. In 1996, we were the first to describe that ART induced apoptosis in cancer cells [40]. This has been corroborated in a plethora of subsequent publications (Table 6, [34,48,50,51,55,57,58,61–63,65,68,71,72,74,81,82,86–90,93–95,97–99,108,111,115,125,126,141–144,148,150,152–154,160,163–189]). Depending on the cell model, ART can induce either the intrinsic, mitochondrial and the extrinsic FAS-receptor-driven pathways of apoptosis [86,87] with up-regulated 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 ART [85].

9.2. Non-apoptotic cell death

ARS-type drugs also induce caspase-independent forms of non-apoptotic apoptosis, such as autophagy (Table 7, [64,73,92,98,99,102,114,124,138,152,155,190–192]). Necrosis and necroptosis have also been reported to be induced by ARS-type drugs (Table 7). While necrosis represents a non-programmed mode of cell death, necroptosis occurs in a programmed manner. Necroptosis frequently serves as cellular defense mechanism upon microbial infection. Other mode of non-programmed cell death represent oncosis (ischemic cell death) and anoikis (anchorage-dependent cell death), which also has been described for ARS-type drugs in tumor cells [52,64,78].

A novel mode of iron-dependent cell death, termed ferroptosis has been recently described [193,194]. Since ferrous iron enhances cytotoxicity of ARS-type drugs towards cancer cells, a

Table 4
DNA damage and repair induced by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
Molt-4 and RTN leukemia	DHA, ART-TF	DHA-resistant RTN cells revealed less X-ray-induced DNA damage than wild-type Molt-4 cells	Park et al. [140]
Epithelial origin:			
PG100 gastric Ca	ARM	Induction of DNA damage	Alcântara et al. [138]
Liver Ca	ARS ART	Induction of DNA damage	Aquino et al. [139]
Ovarian Ca		Induction of DNA double-strand breaks. Rad51↓, homologous recombination repair↓	Wang et al. [141]
Diverse origin:			
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. [81,82,137]
	ART	Induction of double-strand breaks. Involvement of base excision repair, homologous recombination repair and non-homologous end-joining.	Li et al. [135]
	ART	Induction in oxidative DNA damage that resulted in DNA-double strand breaks. Involvement of homologous recombination repair and non-homologous end-joining.	Berdelle et al. [136]

Table 5
Cell cycle arrest induced by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
SP2/0 multiple myeloma	ART	G_0G_1 phase arrest	Li et al. [144]
CCRF-CEM and CEM/ADR5000 leukemia	Artesunic acid	G_0G_1 phase arrest	Horwedel et al. [146]
Mesenchymal origin:			
Osteosarcoma	DHA	G_2M phase arrest; cyclin D1↑, CDC25B↓, cyclin B1↓	Ji et al. [148]
Epidermal origin:			
Ovarian carcinoma	DHA	G_2M phase arrest	Jiao et al. [142]
Ovarian carcinoma	ART	Dose-dependent G_0G_1 or G_2M phase arrests	Greenshields et al. [77]
MCF7 breast Ca	ARS	G_0G_1 phase arrest	Sundar et al. [161]
MCF7 breast Ca	ARS	G_0G_1 phase arrest; cyclin D1↓, cyclin E↓, CDK2↓, CDK4↓, E2F1↓	Tin et al. [59]
	DHA	G_0G_1 phase arrest	Mao et al. [153]
MCF7 and MDA-MB-231 breast Ca	ART	G_2M phase arrest, p21↑	Chen et al. [155]
Hepatoma	ART, DHA	G_0G_1 phase arrest; cyclin D1↓, cyclin E↓, CDK2↓, CDK4↓, E2F1 ↓, p21 ↑, p27↑	Hou et al. [50]
Hepatocellular Ca	DHA	G_2M phase arrest; p21↑, CDC25B↓, cyclin B↓	Zhang et al. [61]
LnCaP lymph node lesion of prostate Ca		G_0G_1 phase arrest; CDK2↓, CDK4↓, pSp1 ↓	Willoughby et al. [146]
BxPC-3 and AsPC-1 pancreas Ca	DHA	G_0G_1 phase arrest; regulation of cyclin E, CDK2↓, CDK4↓, p27↑, p21↑	Chen et al. [51,145]
HCT116 colorectal Ca	DHA	G_0G_1 phase arrest	Lu et al. [115]
HCT116 and HCT116/R colorectal Ca	DHA	G_0G_1 phase arrest, GADD153↑, GRP78↑	Lu et al. [97]
CNE-1 and CNE-2 nasopharyngeal Ca	ARS	G_0G_1 phase arrest; p16↓, CDK4↓	Wu et al. [149]
Nasopharyngeal Ca	DHA	G_0G_1 phase arrest	Huang et al. [160]
A431 epidermoid Ca	ART	G_0G_1 phase arrest; cyclin A1↓, cyclin B1↓, cyclin D1↓, CDK2↓, CDK4↓, CDK6↓	Jiang et al. [150]
GH3 rat pituitary adenoma	ART	G_2M phase arrest	Mao et al. [151]
Ishikawa endometrial Ca	ARS	G_0G_1 phase arrest; CDK2↓, CDK4↓	Tran et al. [156]
Esophageal Ca	DHA	G_0G_1 phase arrest; cyclin E↓, CDK2↓, CDK4↓	Du et al. [152]
Eca109 and Ex9706 esophageal Ca	ART	G0G1 phase arrest; CDC25A↓	Liu et al. [79]
KYSE-150 esophageal Ca	ART	G_0G_1 phase arrest	Shi et al. [159]
SGC-7901, BGC823 and MGC803 gastric Ca	DHA	G_0G_1 phase arrest, p21↑, p27↑, PCNA↓, cyclin E↓, cyclin D1↓	Sun et al. [63]
AGS and MKN74 gastric Ca	ARS	p21↑, p27↑	Zhang et al. [157]
RB-Y79 retinoblastoma	ART	G_0G_1 phase arrest	Zhao et al. [120]
Glioma stem cells	DHA	G_0G_1 phase arrest	Cao et al. [154]
Neuroblastoma	ARS	G_0G_1 phase arrest	Zhu et al. [158]
Caki-1, 786-O and SN12C-GFP-SRLu2 kidney Ca	ART	G_2M phase arrest	Jeong et al. [257]
A549 and H1299 lung Ca	ARS	G_0G_1 phase arrest	Tong et al. [76]
Gallbladder Ca	ARS	G_0G_1 phase arrest; cyclin D1↓, CDK4↓	Jia et al. [71]
Diverse origin:			
55 NCI cell lines	ART	Correlation of G_0G_1 and S phases to IC_{50}	Efferth et al. [85]
Diverse	ART	Both G_0G_1 or G_2M arrest	Steinbrück et al. [147]

role of ferroptosis for their mode of action is apparent (Table 7). The RAS-selective lethal compound erasin and the tyrosine kinase inhibitor sorafenib induced ferroptosis [193,194]. Ferrostatin-1 and deferoxamine are iron-depleting agents that inhibited ferroptosis induction [75,77,195,196].

Ooko et al. (2015) found that the $\log_{10}IC_{50}$ values of 10 ARS derivatives significantly correlated to the microarray-based mRNA expression of 20 out of 30 iron-related genes in 60 NCI cell lines as determined in 218 different microarray hybridization experiments [124]. These genes included those for transferrin (TF), transferrin receptors 1 and 2 (*TFRC, TFR2*), ceruloplasmin (*CP*), lactoferrin (*LTF*) *etc*. Ferrostatin-1 and deferroxamine diminished the activity of DHA.

Table 6
Apoptotic cell death induced by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
KG-1a leukemia	ART	Apoptosis	Efferth et al. [34]
MOLT-4 leukemia	ARS	Induction of apoptosis, but not necrosis	Singh and Lai [108]
Jurkat and CCRF-CEM leukemia	ART	Intrinsic pathway of apoptosis	Efferth et al.7 [88]
HL-60 leukemia	DHA	Induction of apoptosis, p38 MAPK↓	Lu et al. [111]
Jurkat leukemia	DHA	Induction of apoptosis, mitochondrial membrane potential↓, cytochrome C release, caspases↑, Bcl-2↓, Bcl-cL↓, NOXA↑, Bax↑	Handrick et al. [171]
primary acute myeloic and lymphoblastic leukemia cells	DHA	Induction of apoptosis, cytochome C release, caspase↑, Mcl-1↓, MEK/ERK↓	Gao et al. [57]
chronic myeloic leukemia cells	DHA	Induction of apoptosis, BCR/ABL↓, AKT↓, ERK↓, cytochrome C release, caspases 3/9↑	Lee et al. [179]
SP2/0 multiple myeloma	ART	Induction of apotosis, nuclear NF-κB p65↓, IκBβ↑	Li et al. [144]
Multiple myeloma	ART	Induction of apoptosis, MYC↓, Bcl-2↓, caspase 3↑	Holien et al. [178]
Multiple myeloma	ART	Induction of non-caspase apoptosis, mitochondrial membrane potential↓, translocation of A/F and EndoG	Papanikolaou et al. [99]
Ramos non-Hodgkin lymphoma	ART	Extrinsic pathway of apoptosis, YY1↓, Sp1↓, Bid↑	Sieber et al. [87]
Diffuse large B-cell lymphoma	ART	Induction of apoptosis, MYC↓, Bcl-2↓, caspase 3↑	Holien et al. [178]
T-cell lymphoma	DHA	Induction of apoptosis	Wang et al. [125]
P815 murine mastocytoma	ARS	Induction of apoptosis	Tilaoui et al. [67]
SKM-1 myelodysplastic syndrome	ART	Induction of apoptosis	Xu et al. [186]
Mesenchymal origin:			
OSCA2, OSCA16, OSCA50 and D17 canine osteosarcoma	DHA	Induction of apoptosis, caspase 3↑	Hosoya et al. [167]
Osteosarcoma	DHA	Induction of intrinsic and extrinsic apoptosis, caspases 3/8/9↑, FAS↑, Bax↑, Bcl-2↓, NF-κB↓	Ji et al. [148]
Osteosarcoma	DHA	Induction of apoptosis, GSK3β↑	Liu et al. [62]
143 B osteosarcoma	DHA	Induction of apoptosis	Liu et al. [79]
Osteosarcoma	DHA	Induction of apoptosis	Tang et al. [185]
Rh30 and RD rhabdomyosarcoma	DHA	Induction of apoptosis	Odaka et al. [181]
Epithelial origin:			
IHGK oral squamous cell Ca	ARS	Induction of apoptosis, Bax↑, Bcl-2↓	Yamachika et al. [163]
C6 rat glioma	DHA	Induction of apoptosis, HIF-1 α↓	Huang et al., [164]
Glioma stem cells	DHA	Induction of apoptosis, p-AKT↓, caspase 3↑	Cao et al. [154]
C6 rat glioma	DHA	Induction of apoptosis	Kim et al.[126]
SHG44 glioma	ART	Induction of apoptosis	Lian et al. [184]
Ovarian carcinoma	DHA	Induction of apoptosis Bcl-2↓, Bcl-xL↓, Bax↑, Bad↑	Jiao et al. [142]
SPC-A-1 lung Ca	DHA	Induction of apoptosis, survivin↓	Mu et al. [165]
PC-14 lung Ca	DHA	Induction of apoptosis, Ca^{2+}↑, p38 activation	Mu et al. [166]
ASTC-a-1 lung Ca	DHA	Induction of apoptosis, mitochondrial membrane potential↓, caspase 3↑	Lu et al. [258]
Lewis murine lung Ca	DHA	Induction of apoptosis	Zhou et al. [170]
ASTC-a-1 lung Ca	DHA	Induction of intrinsic and extrinsic apoptosis, mitochondrial membrane potential↓, cytochrome C release, caspases 3/8/9↑, Bid↑	Lu et al. [88]
A549 lung Ca	ART	Induction of apoptosis, EGFR↓, AKT↓, ABCG2↓	Ma et al. [256]
A549 and ASTC-a-1 lung Ca	DHA	Induction of apoptosis, induction of endoplasmic reticulum stress, Bim↑	Chen et al. [176]
ASTC-a-1 and A549 lung Ca	ART	Induction of intrinsic apoptosis, release of Smac and AIF, Bak↑, VDAC2↓, Bim↑	Zhou et al. [93]
A549 lung Ca	ARS	Induction of apoptosis, mitochondrial membrane potential↓, Bid cleavage, release of SMAC and AIF, caspases 3/8/9↑	Gao et al. [94]
A549 lung Ca	ART	Induction of apoptosis and autophagy, accumulation of acidic vacuoles, cytochrome C release, caspase 3↑	Ganguli et al. [95]
HepG2, Huh-7, BEL-7404 and, Hep3 B liver Ca	ART, DHA, ARM, ARS	Induction of apoptosis, Bax/Bcl-2 ratio↑, PARP↑, MDM2↓	Hou et al. [50]
HepG2 liver Ca	DHA	Induction of apoptosis, Ca^{2+}↑, GADD153↑,BaX↑, Bcl-2↑	Gao et al. [89]
Hepatocellular Ca	DHA	Induction of apoptosis, cytochrome C release, caspases 3/9↑, Mcl-1↓, NOXA↑, Bax↑, Bak↑, Bim↑,	Qin et al. [183]
BxPC-3 and ASPC-1 pancreas Ca	DHA	Induction of apoptosis, nuclear NF-κB p65↓, Bax↑, Bcl-2↓, caspases 3/9↑	Chen et al. [51,143]
MiaPaCa-2 and BxPC-3 pancreas Ca	ART	Induction of apoptosis, caspases 3/7↑, TOPO2A↓	Youns et al. [169]
BxPC-3 pancreas Ca	DHA	Induction of apoptosis, Bcl-2↓, Bax↑	Aung et al. [174]
R/N pancreas Ca	ARS	Induction of apoptosis	Noori et al. [98]
Pancreas Ca	DHA	Tumor growth↓, involvement of 5 microRNAs *in vitro*	Li et al. [74]
Melanoma	ART	Induction of apoptosis in melanoma cells of ret-transgenic mice	Ramacher et al. [168]
A375, G361 and LOX melanoma	DHA	Induction of apoptosis, p53 phosphorylation, NOXA↑	Cabello et al. [90]
Prostate Ca	DHA	Induction of intrinsic and extrinsic apoptosis, P13-K/AKT and ERK pathways↓, death receptor DR5↑	He et al. [172]

Table 6 (*Continued*)

Cell line	Drug	Effect	Reference
PC-3 prostate Ca	DHA	Induction of apoptosis, 86 deregulated genes	Xu et al. [188]
Neuroblastoma	ART	Induction of apoptosis, role of glutathione mechanism	Michaelis et al. [173]
MCF-7 and MDA-MB-231 breast Ca	ART	Induction of apoptosis; resistance by NF-κB↑, Bcl-2 ↑ and Bax↓	Bachmeier et al. [55]
Breast Ca	DHA	Induction of intrinsic apoptosis, cytochrome C release, caspases 8/9↑, Bid activation, Bim↑, Bcl-2↓	Mao et al. [153]
HCT-116 colorectal Ca	DHA	Induction of apoptosis endoplasmic reticulum stress, GRP78↑, GADD153↑	Lu et al. [115]
HCT-116/R colorectal Ca	DHA	Induction of apoptosis, heat shock proteins↑	Lu et al. [177]
Colorectal Ca	DHA	Induction of apoptosis, mitochondrial membrane potential↓, caspases 3/8/9↑, cytochrome C release, AIF translocation	Lu et al. [97]
HeLa cervical Ca	ART	Induction of extrinsic apoptosis, survivin↓, XIAP, AKT inactivation, inhibition of TRAIL-induced transcriptional activation of NF-κB	Thanaketpaisarn et al. [175]
HeLa and Caski cervical Ca	DHA	Induction of apoptosis, RKIP↑, Bcl-2 ↓	Hu et al. [180]
ME-180 cervical Ca (HPV-39 infected)	ARS	Induction of apoptosis, decreased telomerase activity, hTR↓, hTERT↓, HPV-39 E6 and E7↓	Mondal and Chatterji [182]
A431 epidermoid Ca	ART	Induction of intrinsic apoptosis	Jiang et al. [150]
A431 epidermoid Ca	DHA	Induction of apoptosis	Hui et al. [187]
Esophageal Ca	DHA	Induction of apoptosis, Bax↑, Bcl-2↑, Bcl-xL↓, procaspase-3↓, caspase-9↑	Du et al. [152]
Eca109 and Ec9706 esophageal Ca	ART	Induction of apoptosis, mitochondrial membrane potential↓, Bax↑, Bcl-2↑, caspase-3↑	Liu et al. [79]
CNE-2 nasopharyngeal Ca	DHA	Induction of apoptosis	Huang et al. [160]
SGC-7901, BGC823 and MGC803 gastric Ca	DHA	Induction of apoptosis, Bcl-2↓, caspase 9↑, PARP↑	Sun et al. [63]
Gastric Ca	ART	Induction of intrinsic apoptosis, COX2↓, Bax↑, Bcl-2↓, mitochondrial membrane potential↓, caspases 3/9↑	Zhang et al. [259]
BGC-823 gastric Ca	DHA	Induction of apoptosis, Bax↑, Bcl-2↓, BAX, caspases 3/9↑, PARP↑,	Zhang et al. [78]
BSR hamster kidney adenocarcinoma	ARS	Induction of apoptosis	Tilaoui et al. [67]
SCC25 squamous cell Ca	ART	Induction of apoptosis and autophagy, accumulation of acidic vacuoles, cytochrome C release, caspase 3↑	Ganguli et al. [95]
MDA-MB-231 breast Ca	ART	Induction of apoptosis and autophagy, accumulation of acidic vacuoles, cytochrome C release, caspase 3↑	Ganguli et al. [95]
Gallbladder Ca	ART	Induction of apoptosis, miR-16↑, COX-2↓, PGE2↓	Zuo et al. [68]
Gallbladder Ca	ARS	Induction of apoptosis, mitochondrial membrane potential↓, cytochrome C release, caspases 3↑	Jia et al. [71]
Head and neck squamous cell Ca	DHA	Induction of apoptosis	Jia et al. [72]
Diverse origin:			
55 NCI cell lines	ART, ARE, ARM	Correlation of microarray-based apoptosis-regulating genes to IC_{50} values.	Efferth et al. [81,85]

10. Anti-angiogenesis

Phytochemicals are frequently multi-target specific molecules compared to targeted synthetic small molecule inhibitors [197,198]. Therefore, ARS-type drugs may exert their anti-cancer effects by addressing multiple rather than single mechanisms. In addition to the modes of action described above, ARS-type drugs inhibited tumor angiogenesis (Table 8, [46,47,54,66,74,170,174,182,199–209]). Diverse models provided convincing evidence for the involvement of angiogenesis in their mechanism of action, *e.g.* blood vessel endothelial cells (HUVEC), the corioallantoic membrane (CAM) assay, or human xenograft tumors in nude mice. The secretion of angiogenic factors (*e.g.* VEGF, KDR/flk-1, VEGFR2) by tumor cells was inhibited by ARS treatment. ART also inhibited angiogenesis in matrigel plug assays *in vivo* [48]. The microarray-based mRNA expression of 30 of our 89 angiogenesis-related genes in the NCI cell line panel was significantly correlated with the $\log_{10}IC_{50}$ values for 8 ARS-type compounds [202].

The anti-angiogenic potential of various ARS derivatives were investigated in a Zebrafish model and compared to results obtained by molecular docking and quantitative structure relationship (QSAR) analyses [210,211]. 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 experimentally validated set of control data was then used to predict angiogenic activity in a test set of 52 other ARS derivatives by performing molecular docking VEGFR1, VEGFR2, and VEGFA. The best binding affinities were observed for VEGFR1, and novel candidate compounds were identified for further investigation [211].

11. Targeting signal transduction

ARS-type drugs affect several important signaling routes in cancer cells (Table 9, [49,62,69–72,76,78,80,169,179,181,187,188,212–228]). The compounds inhibited the Wnt/β-catenin pathway by down-regulation of β-catenin and translocation of β-catenin from the nucleus to the cell membrane. ARS-type drugs also inhibited epidermal growth factor receptor as well as BCR/ABL signalin. Cancer-related transcription factors were shut down, *e.g.* mTOR, MYC/MYX, NF-κB, AP-1 (FOS/JUN), CREB etc. Important regulators of invasion and metastasis were down-regulated by ART, such as ubiquitous plasminogen activator (u-PA) and metalloproteinases (MMP-1, −2 and −7) [57,221].

An interesting target of ARS-type drugs has first been desribed in the context of malaria therapy. It was the translationally controlled

Table 7
Non-apoptotic cell death induced by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
K562 leukemia	DHA	Induction of autophagy, LC3-II↑	Wang et al. [92]
Multiple myeloma	ART	Induction of non-caspase apoptosis, depolarization of mitochondrial membrane, translocation of AIF and EndoG	Papanikolaou et al.[99]
Epidermal origin:			
Panc-1, BxPC-3 and CFPAC-1 pancreas Ca	ART	Induction of oncosis, depolarization of mitochondrial membrane	Du et al. [52]
Pancreas Ca	DHA	Induction of autophagy, beclin1↑, JNK pathway↑	Jia et al. [96]
PDAC pancreas Ca	ART	Induction of ferroptosis	Eling et al. [102]
MCF-7 breast Ca	ART	Induction of autophagy, inhibition of autophagosome turnover, perinuclear clustering of autophagosomes, early and late endosomes and lysosomes	Hamacher-Brady et al. [114]
MCF7 and MDA-MB-231 breast Ca	ART	Induction of autophagy, beclin1↓, stimulation of LC3 stimulation, p21↑	Chen et al. [155]
PG 100 gastric Ca	ARM	Induction of necrosis	Alcântara et al. [138]
RT4 schwannoma	ART	Induction of necroptosis	Button et al. [190]
PDAC panceas Ca	ART	ferroptosis activation	Eling et al. [102]
Head and neck squamous cell Ca	ART	Cell death blocked by ferroptosis inhibitor	Roh et al. [75]
Ovarian Ca	ART	Cell death blocked by ferroptosis inhibitor	Greenshields et al. [77]
Diverse origin:			
Diverse	DHA	Induction of autophagy, NF-κB↓	Hu et al. [191]
Diverse	DHA	Induction of autophagy by p8endoplasmic reticulum stress-related ATF4 and CHOP↑	Chen et al. [192]
60 NCI cell lines	ART, ARS, ARE, ARM	Cell death blocked by ferroptosis inhibitors. Correlation of iron-regulating genes with IC_{50} values of artemisinins	Ooko et al. [124]

Table 8
Inhibition of angiogenesis by ARS-type drugs.

Cell line	Drug	Effect	Reference
Hematopoietic origin:			
RPMI8226 multiple myeloma	DHA	VEGF secretion↓, neovascularization in CAM assay↓	Wu et al. [203]
RPMI8226 multiple myeloma	ART	VEGF and Ang-I secretion↓, neovascularization in CAM assay↓	Chen et al. [205]
K562 leukemia	ART	VEGF secretion *in vitro* and *in vivo*↓	Zhou et al. [204]
Mesenchymal origin:			
HUVEC endothelial cells	ART	Migration in scratch assay↓, microvessel tube-like formation on collagen gel↓	Chen et al. [199]
HUVEC endothelial cells	ART	Tumor growth↓	Chen et al. [48]
HUVEC endothelial cells	DHA	VEGF-binding to its receptors↓, Flt-1↓, KDR/flk-1↓, neovascularization in chicken chorioallantoic membrane (CAM) assay↓	Chen et al. [200]
HUVEC endothelial cells	DHA	VEGFR2↓, nuclear translocation of NF-κB↓, IκB alpha↑	Dong et al. [208]
HUVEC endothelial cells	DHA	ERK1/2↓, ERK1/2 phosphorylation, FOS↓, MYC↓	Dong et al. [211]
Epithelial origin:			
HO-8910 ovarian Ca (*in vivo*)	ART	Tumor growth↓, VEGF↓, KDR/flk-1↓,	Chen et al. [46]
C6 rat glioma (*in vivo*)	ARM	Tumor growth↓, microvessel density↓	Wu et al. [54]
BxPC-3-*RFP* pancreas Ca	DHA	VEGF *in vivo*↓	Aung et al. [174]
Murine Lewis lung Ca	DHA	KDR/flk-1↓	Zhou et al. [170]
PANC1 and BxPC-2 pancreas Ca	DHA	Inhibition of angiogenesis, involvement of microRNAs	Li et al. [74]
HepG3 and BWTG3 liver Ca, Diethylnitrosamine-induced tumors	ART	VEGF *in vitro* and *in vivo*↓	Vandewynckel et al. [66]
ME-180 cervical Ca	ARS	VEGF↓	Mondal and Chatterji [182]
Diverse origin:			
	ART	Blood vessel formation *in vivo* using the matrigel plug assay↓	Dell-Eva et al. [47]
	ART	Neovascularization in CAM assay↓	Huan–Huan et al. [207]
60 NCI cell lines	ARS, ART, ARE etc.	mRNA expression of iron-related genes correlated with IC_{50} values	Anfosso et al. [202]
55 NCI cell lines	ART	mRNA expression of the angiogenesis promoting factor ITGB1 correlated with IC_{50} values	Sertel et al. [206]
HUVEC endothelial cells, BxPC-3 prostate Ca	DHA	Growth and tube formation↓, NF-κB binding↓, VEGF↓, IL8↓, COX2↓, MMP9↓, microvessel density *in vivo*↓	Wang et al. [207]

tumor protein (TCTP) [229–231]. The crystal structure of *Plasmodium falciparum* TCTP (PfTCTP) was determined and binding sites of ARS were determined in direct neighborhood to amino acids 19–46, 108–134 and 140–163. TCTP is involved in the regulation of proliferation and can be found throughout all eukaryotic organisms from protozoa to plants and mammals [232].

In cancer, TCTP regulates cell cycle transition, apoptosis, calcium homeostasis, and cytoskeleton. The protein is also involved in a phenomenon, termed tumor reversion, which is characterized

Table 9
Effect of ARS-type drugs on signal transduction.

Cell line	Drug	Effect	Reference
Wnt/β-catenin pathway:			
RAW 264.7 murine macrophages	ART	Involvement of cAMP-mediated and Wnt/β-catenin signaling pathways.	Konkimalla et al. [213]
SKM-1 myelodysplastic syndrome	ART	Inhibition of Wnt/β-catenin signaling pathway; translocation of β-catenin from nucleus to cytoplasm	Xu et al. [186]
Osteosarcoma	DHA	β-catenin↓ because of increased catalytic activity of GSK3β, Wnt/β-catenin signaling↓	Liu et al. [62]
HT-29 colorectal Ca	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. [49]
HT-29 colorectal Ca	ART	Membraneous translocation of β-catenin, E-cadherin↑, reversion of EMT	Li et al. [212]
Lung Ca	ARS, ART, DHA	Inibition of Wnt/β-catenin signaling pathway	Tong et al. [76]
A431 epidermoid Ca	DHA	Inhibition of Wnt/β-catenin signaling pathway	Hui et al. [187]
Receptor and downstream kinase signaling:			
K562 leukemia	DHA	BCR/ABL↓, downstream signal transducers (AKT and ERK1/2 tyrosine kinase activity↓, NF-κB protein expression↓	Lee et al. [179,215]
Acute myeloic leukemia	ART, DHA	preferential killing of cells with FLAT3-ITD mutations	Drenberg et al. [69]
BGC-823 gastric Ca	DHA	Phosphorylation of ERK1/2, JNK1/2 and p38MAPK↑	Zhang et al. [78]
Gallbladder Ca	ARS	Inhibition of ERK1/2 pathway	Jia et al. [71]
Head and neck squamous cell Ca	DHA	Inhibition of Jak2/STAT3 pathway	Jia et al. [72]
MDA-MB-231 breast cancer induced ostelysis	DHA	Inhibition of AKT/SRC pathway	Feng et al. [88]
Liver carcinogenesis	ART	Inhibition of IL6-JAK-STAT pathway	Ilamathi et al. [70]
55 NCl 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. [214]
Diverse	ARS, ART, DHA etc.	A network pharmacology approach revealed five major pathways: PI3 K/AKT, T cell receptor, Toll-like receptor, TGF-β and insulin signaling pathways	Huang et al. [216]
mTOR pathway:			
Rh30 and RD rhabdomyosarcoma	DHA	mTOR signaling pathways↓	Odaka et al [181]
SHSY5Y neuroblastoma	ARS	AMP kinase signaling↑, mTOR/p70S6 K/p S6 signaling↓	Tan et al. [217]
Transcription factors:			
RAW 264.7 macrophages	DHA	PMA-induced COX-2 expression↓ 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. [219]
60 NCI cell lines	ART	Promoter binding motif analyses of differentially expressed genes identified MYC/MAX as transcriptional regulators	Sertel et al. [218]
Metastatic signaling:			
HT-1080 fibrosarcoma	DHA	Cell invasion and migration↓, MMP-9↓, MMP-2↓. Inhibition of MMP-9 expression by NF-κB, inhibition of MMP2 by MT1-MMP. No effect on TIMP-1 and TIMP-2	Hwang et al.[220]
H1395, A549, LXF289, H460, Calu3, and H1299 lung Ca	ART	u-Pa↓, MMP2↓, MMP7↓, AP-1↓, NF-κB↓	Rasheed et al. [221]
CaSki and HeLa cervical Ca	ART	HOTAIR↓, COX-2↓	Zhang et al. [228]
Translationally Controlled Tumor Protein:			
	DHA	Binding to Fortilin/TCTP, ubiquitination↓, proteasome-dependent shortening of TCTP half-life. TCTP-knockdown cells were DHA-resistant, TCTP-transfectant cells were more DHA-sensitive	Fujita et al. [222]
A549 lung Ca	DHA	*TCTP* mRNA↑, but TCTP protein↓. Increased TCTP protein secretion	Liu et al. [223]
NF1-deficient Schwann cells, MPNST neurofibromatosis type 1 (NF1)	ART	Binding and degradation of TCTP, MPNST↓ but not normal Schwann cells. TCTP level inversely correlated with ART sensitivity	Kobayashi et al. [224]
Other mechanisms:			
RAW 264.7 murine macrophages	ART	NO↓	Konkimalla et al. [213]
T-cells	DHA	Th cell differentiation↓, TGF-βR/Smad-dependent Treg generation↑, mTOR pathway↓.	Zhao et al. [226]
HepG2 liver Ca	ART	NO↓, heme-harboring NOS↓	Zeng and Zhang [225]
MiaPaCa-2 and BxPC-3 pancreas Ca	ART	TOPO2A↓	Youns et al. [169]
PC3 pancreas Ca	DHA	Aminacyl-tRNA biosynthesis and metabolic pathways	Xu et al. [188]
A549 and H1975 lung Ca	DHA	Inhibition of Warburg effect; inhibition of GLUT1 translocation to plasma membrane	Jiang et al. [227]

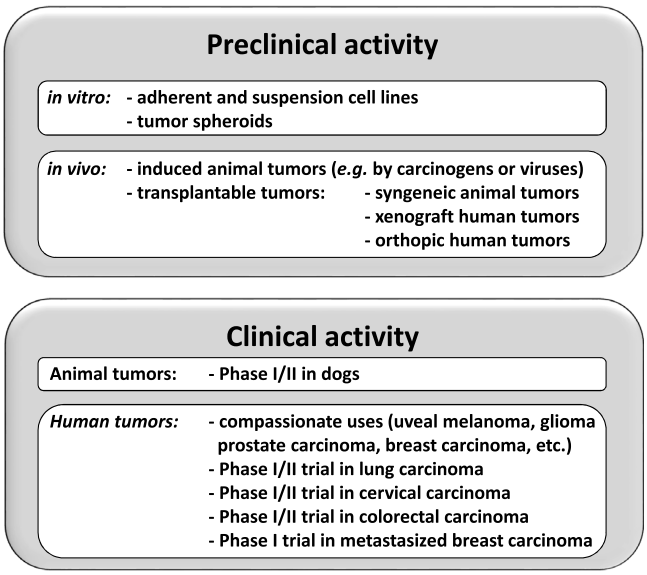

Fig. 3. Preclinical tumor models and clinical tumor types used for treatment with artemisinin and its derivatives.

by the inhibition of tumor transformation. This ultimately leads to the reversion of cancer cells back to normal cell states [233,234]. Recently, a strategy to identify ARS-interacting target proteins in cancer cells has been described [235]. This approach is based on the combination of experimental and bioinformatical techniques. Among the identified proteins was also TCTP [235].

The facts that TCTP is targeted by ARS, and TCTP is involved in cellular differentiation and tumor reversion opens the possibility to treat tumors not only by classical cytotoxic approaches as done in the past decades, but also by novel strategies based on differentiation therapy. This may represent a promising approach, because differentiation therapy halts tumor growth without exerting cytolytic effects. Hence, the well-known severe toxicities of clinically established cytotoxic chemotherapy may not be seen with this kind of tumor treatment. Differentiation therapy with all-trans-retinoic acids is already well-studied for the treatment of acute promyelocytic leukemia [236,237]. Whether ARS-type drugs are useful for novel differentiation therapy approaches and tumor reversion therapy warrants more detailed analyses in the future.

12. Preliminary clues for clinical anti-cancer activity

In light of the huge amount of preclinical data acquired during the past two decades on the anticancer effects of ARS-type drugs, the time has come to investigate their clinical activity (Fig. 3). This is an attractive issue, not only because novel anticancer drugs are urgently required, but also owing to the fact that ARS-type drugs are known to be well tolerated, if applied for malaria treatment [238]. Therefore, the question arises about their tolerability in cancer patients.

12.1. Veterinary tumors

As pets such as dogs and cats spontaneously develop tumors in a fashion similar to human beings, the clinical testing of ARS-type drugs is not only relevant for therapy of human tumors, but also for veterinary medicine.

A clinical trial with ART was conducted in 23 dogs with non-resectable tumors [239]. ART was administered for up to 385 days at a dosage of 651–1178 (median 922) mg/m^2. Concerning the safety profile, neurological or cardiac toxicities were not visible. Seven dogs did not show any adverse effects. In another 16 dogs fever, as well as transient hematological or gastrointestinal toxicities were observed. One dog died from pneumonia, which was probably not related to ART treatment. Blood plasma levels of ART and DHA were well detectable 2 h after application with concentrations close to 1 μM, but fell below the detection limit within 8–12 h. Regarding efficacy, one long-lasting complete remission of the tumor and 7 short-term stabilizations of the disease were recorded.

In another clinical trial, 24 dogs were orally administered for 21 days with ARS at low-continuous (3 mg/kg/24 h) or high-dose intermittent doses (three doses of 45 mg/kg/6 h repeated for 1 wk). The most frequent side effect was anorexia (in 11% of the low-dose group and 29% of the high-dose group). Both application schemes were well tolerated in dogs, but resulted in low bioavailability. The authors concluded that parenteral administration should be considered for future studies [240].

Instead of chemically pure ARS or ART, we applied capsules of Herba Artemisiae annuae powder to pets suffering from sarcoma [241]. The standard tumor therapy by surgery was supplemented by adjuvant add-on therapy with *A. annua*. One cat and one dog with fibrosarcoma survived 40 and 37 months, respectively, without tumor relapse. Two other dogs with fibrosarcoma or hemangioendothelial sarcoma revealed complete remission and survived for further 39 and 26 months, respectively. Surgical removal of the tumor as standard therapy for these types of tumors usually resulted in survival times between 7–12.2 months. It is noteworthy that *A. annua* capsules increased the survival times of the pets and were well tolerated without obvious side effects. In this context, it has to be mentioned that ARS is not the only bioactive compound in *A. annua*. Various other phytochemicals are also known to exert cytotoxicity against tumor cells [242]. It is reasonable to consider *A. annua* as "natural combination therapy", which might be even more effective against tumors than ARS alone.

12.2. Case reports of human cancer patients

Three patients responded well to ARS-type drugs [243]. A 47-year-old metastasized stage-4 breast cancer revealed tumor regression in computer tomography upon ARS treatment. Another breast cancer patient made comparable experiences. Another 47-year-old patient with terminal hepatocellular carcinoma and abdominal ascites was still alive 2.5 years after taking ARS [243]. Another case report described the ART therapy of a laryngeal squamous cell carcinoma patient [244].

We reported on two uveal melanoma cases upon compassionate treatment with ART after ineffective standard chemotherapy [245]. ART was well tolerated in both patients. One patient received fotemustine plus ART and reached a temporary response, although the tumor was progressing under prior fotemustine therapy alone. This patient died 23 months after entry in stage 4 disease. The second patient reached disease stabilization after application of dacarbazine and ART. Subsequently, metastases in lung and spleen appeared. This patient was alive at the time point of publication of this case report, which was 47 months after first diagnosis. ART was well tolerated without side effects in both patients. The results of these compassionate therapies with ART are remarkable in light of the usual median survival times for uveal melanoma in the range of two to five months.

Longitudinal observations on the efficacy of *A. annua* in a prostate carcinoma patient were reported [246]. The tumor was staged as pT3bN1M1 with a Gleason score 8 (4 + 4)) by imaging techniques (MRT, scintigraphy, SPECT/CT). The patient presented with a prostate specific antigen (PSA) blood level of >800 μg/L. After short-term intake of bacalitumide (50 mg/d for 14 days) and long-term oral therapy 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. Blood PSA

and ostase levels increased 7 months later, indicating tumor recurrence and skeletal metastases. *A. annua* capsules were substituted by ART injections (2 × 150 mg twice weekly *i.v.*), but fatal tumor recurrence was not prevented anymore. 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. High immunohistochemical expression of MYC, TFR, and VEGFC in the tumor biopsy corresponded with high expression of these markers in the ARS-sensitive PC-3 cells compared to ARS-resistant DU-145 cells. In summary, long-term treatment with *A. annua* capsules combined with short-term bicalitumide treatment led to temporary regression of advanced metastasized prostate carcinoma.

Because of the aggressiveness of glioblastoma, most patients suffering from this tumor cannot be cured. Patients are frequently desparating seeking for compassionate use of non-approved drugs and drug combinations. Although ARS-type drugs are generally considered as safe and well tolerated, their combination with other medications may lead to adverse events. A glioblastoma multiforme patient treated with a combination of temozolomide, ART and Chinese herbs (*Coptis chinensis, Siegesbeckia orientalis, Artemisia scoparia, Dictamnus dasycarpus*) suffered from reversible hepatotoxicity [247]. While these drugs alone may bear a minor risk for hepatotoxicity, this specific combination increased liver enzyme activities in this patient. Another glioblastoma patient was treated by an alternative practitioner with a combination of dichloroacetate and ART. The patient showed clinical and laboratory signs of severe liver damage and bone marrow toxicity (leukopenia, thrombocytopenia) and died a few days later [248]. The compassionate use of dichloroacetate/ART combination therapy outside of clinical trials cannot be recommended. These drastic examples illustrate that even if ART alone is considered to be well tolerated, non-approved combinations with medications from complementary and alternative medicine should be avoided.

12.3. Clinical trials

A clinical trial in advanced non-small cell lung cancer reported on efficacy and toxicity of the standard combination vinorelbine and cisplatin with or without intravenous ART injections (120 mg) for 8 days [249]. Each treatment group consisted of 60 patients. Statically significant improvements in short-term survival were measured. 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 ART-treated patients (24 weeks) was significantly longer than that of the control group (20 weeks). ART was well tolerated and increased toxicity was not observed.

Ten cervical carcinoma patients (stage III or IV) were treated with DHA for 28 days at a cancer hospital in Treichville (Ivory Coast) [250]. Clinical symptoms of the disease (vaginal discharge and pain) disappeared within the first three weeks in all patients with a median time of 7 days. Tolerable adverse events (headache and abdominal pain) but no severe toxicity (grade 3 or 4) were observed. Immunohistochemistry revealed a decrease of expression of the tumor suppressor p53, the oncogene EGFR, and Ki-67 as nuclear proliferation marker, as well as the number of CD31-positively stained blood vessels during DHA treatment, while transferrin receptor expression increased. The tumors of six patients relapsed after four to 8 months (average six months). Two patients passed away died after remisiion for 6–7 months. Four relapsed tumors were treated a second time with DHA for 28 days, and clinical remission was achieved. Twelve to 13 months after the first DHA treatment, two of these patients died due to renal insufficiency. At the time point of publication of this study, the other 6 patients revealed a median time of 9 months (range 2–24 months) after their first DHA therapy. The usual survival prognosis of metastasized cervix cancer patients in this hospital is about four months. This prognosis is similar to those in other African cancer clinics, *e.g.* gynecological centers in Kigali, Rwanda and Nairobi, Kenya. This clinical phase I/II pilot trial with DHA was encouraging regarding reduction of clinical symptoms of advanced cervical carcinoma, tolerability and effects on survival time of patients.

A single center, randomized, double-blind, placebo-controlled trial has been performed in 23 colorectal carcinoma patients at St. Georg's (University of London, London, UK) [251]. After diagnosis and prior to surgery, patients were administered either 14 days with oral ART (200 mg; n = 12) or placebo (n = 11). The outcome parameters were clinical survival as well as immunohistochemical assessment in terms of apoptosis rates (measured by the TUNEL assay) and the expression of several tumor markers (VEGF, EGFR, c-MYC, CD31, Ki67 and p53). Twenty out of 23 patients (ART = 9, placebo = 11) completed the trial protocol. Apoptotic cell fractions of >7% were observed in 67% of the ART-treated group and 55% of the placebo-treated patients. Bayesian analysis revealed that the probabilities of ART treatment reduced Ki67 and increased CD31 expression with factors of 0.89 and 0.79, respectively. Median follow up for 42 months showed that one ART-treated patient and six placebo-treated patients developed refractory tumors. In summary, ART showed anticancer activity in colorectal carcinoma and was generally well tolerated.

Pharmacokinetics of ART and DHA were determined in 23 metastatic breast cancer patients during daily oral administration of ART (100, 150, or 200 mg) for a time period of more than three weeks [252]. The estimated DHA saliva/plasma ratio was in agreement with reported free plasma levels of DHA. Tumor response to ART and survival times of patients were not reported. Since binding of ARS-type drugs to albumin in the blood may affect therapeutic efficacy, we investigated ARS-albumin binding *in vitro* under near physiological conditions [253]. The measured alterations in enthalpy and entropy upon drug binding speak for hydrophobic forces involved in ARS and DHA binding, whereas ART binding was regulated by both hydrophilic and hydrophobic forces. Molecular docking studies demonstrated how slight modifications in the chemical structure of a drug considerably influenced drug binding to its target protein [253].

The same 23 metastatis breast cancer patients mentioned above [252] have been investigated for tolerability of ART in a phase I trial [254]. Four patients had ART-related non-severe adverse events of the auditory system. Four patients had adverse effects of the vestibular system. One of them was severe, but reversible.

In conclusion, ARS and its derivatives DHA and ART are active against cancer cells *in vitro* and *in vivo*. Like many other natural products or derivatives from them [197]. ARS-type drugs reveal multi-specific modes of actions with multiple targets [39,40,235,255]. A wealth of publications appeared during the past two decades on the anticancer activity of ARS and its derivatives, indicating that this class of compounds represent attractive candidates for cancer therapy. This point of view is supported by the available, still preliminary clinical data. Further larger scale clinical phase II and III clinical trials are necessary to provide more compelling evidence on the suitability of ARS its derivatives in clinical oncology.

Conflict of interest

The author declares that there is no conflict of interest.

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