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Therapeutic and Toxicological Inhibition of Vasculogenesis and Angiogenesis Mediated by Artesunate, a Compound with Both Antimalarial and Anticancer Efficacy

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1. Introduction

Artemisinin (ART) and its derivatives, the lactonic sesquiterpenoid compounds, were discovered in China. A crude extract of the wormwood plant *Artemisia annua* (qinghao) was first used as an antipyretic 2000 years ago, and its specific effect on the fever of malaria was reported in the 16th century (Hsu, 2006). The active constituent of the extract was identified and purified in the 1970s, and named qinghaosu, or ART. Although ART proved effective in clinical trials in the 1980s, a number of semi-synthetic derivatives were developed to improve the drug's pharmacological properties and antimalarial potency (Li et al., 2007a).

ART and its active derivatives have been widely used as antimalarial drugs for more than 30 years, and they have also been shown recently to be effective in killing cancer cells. Artesunate (AS) and its bioactive metabolite, dihydroartemisinin (DHA), have been the topic of considerable research attention in recent years for both indications. The key structural feature in all of the ART-related molecules that mediates their antimalarial activity, and some of their anticancer activities, is an endoperoxide bridge. ART- induced apoptosis, or programmed parasite death, is a highly ordered form of parasite suicide affecting both mature and immature parasites. Apoptosis is widely believed to be the mechanism by which ART therapy rapidly kills malaria parasites. A number of research studies have shown that the mechanism of ART embryotoxicity appears to be associated with ART- driven inhibition of fetal hematopoiesis and vasculogenesis. Specifically, higher drug levels of ART have been shown to affect erythroblasts, endothelial cells and cardiovascular cells in the early embryo (Clark et al., 2004; 2008b, White et al., 2006).

Of the available derivatives, AS has the most favorable pharmacological profile for use in ART-based combination treatment of uncomplicated malaria and intravenous therapy of severe malaria (Li and Weina, 2010a). The presence of a hemisuccinate group in the molecule confers water solubility and relatively high oral bioavailability. In a clinical trial of oral and injectable AS (Batty et al, 1998a), the duration of the lag phase in the parasitemia curve was shown to be 1.92-2.81 hrs, which is much shorter than the lag phase of the other 4 ART drugs (4.03-8.89 hrs) suggesting the parasiticidal effect of AS is very rapid. In addition, the time to clear half the parasitemia (PC_{50}) after AS dosing was 3.18-8.48 hrs, which was

much shorter than the PC_{50}s of the other ARTs which ranged from 10.05-19.08 hrs (Li et al, 2007b). AS is rapidly and quantitatively converted *in vivo* to the potent active metabolite, DHA, which was originally obtained by sodium borohydride reduction of ART, an endoperoxide-containing sesquiterpene lactone (Figure 1).

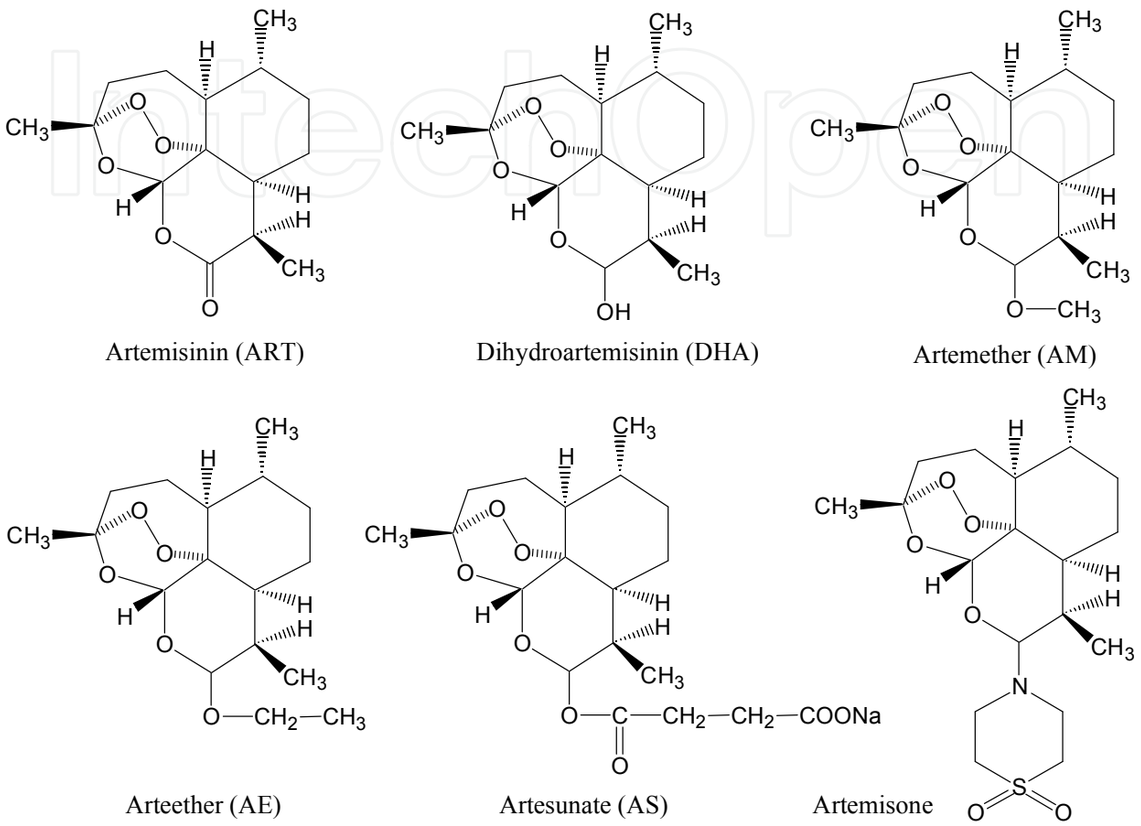

Fig. 1. Chemical structures of artemisinin (ART) and its five derivatives, dihydroartemisinin (DHA), artemether (AM), arteether (AE), artesunate (AS) and artemisone

The effectiveness of AS has been mostly attributed to its rapid and extensive hydrolysis to DHA (Batty et al., 1998b; Davis et al., 2001; Li et al., 1998; Navaratnam et al., 2000). However, due to its poor solubility in water or oils, DHA has only been formulated as an oral preparation and has been used primarily as a semisynthetic compound for derivatization to the oil soluble drugs, artemether and arteether, and the water soluble drug, AS. DHA has similar potency to AS (Li et al., 2007c) and is 3-5 fold more active and more toxic than the other ART derivatives (Li et al., 2002; McLean et al., 1998). It can completely inhibit parasite growth within 2-4 hours, and is the only ART derivative with activity against all asexual blood stage parasites (Skinner et al., 1996).

In recent years, the *in vitro* and *in vivo* anticancer activities of ART have been reported in a number of studies. ART has inhibitory effects on cancer cell growth and also inhibits angiogenesis. Several studies have revealed that ART inhibits the growth of many transformed cell lines and has a selective cytotoxic effect. In one study, ART was shown to be more toxic to cancer than normal cells. In this work, a synthetic compound composed of ART linked to holotransferrin showed enhanced potency and selectivity in killing human leukemia cells. The

cytotoxic effect of the ART-holotransferrin compound is likely due to the high concentration of transferrin receptors in cancer cells that resulted in increased influx of the ART-holotransferrin compound compared to normal cells. Finally, ART possesses an antiangiogenic activity. Angiogenesis, occurring through the proliferation and migration of endothelial cells, is a very important element of tumor development (Buommino et al., 2009; Nakase et al., 2007).

Treatment with AS or DHA can inhibit angiogenesis induced by human multiple myeloma RPMI8226 cells (Chen et al, 2010a;Wu et al, 2006). AS and DHA have been shown to inhibit the growth of Kaposi's sarcoma cells, a highly angiogenic multifocal tumor, and both drugs induced apoptosis in human umbilical vein endothelial cells (Chen et al., 2004a; Dell'Eva et al., 2004). AS and DHA also lowered vascular endotherial growth factor (VEGF) expression and VEGF receptor KDR (kinase insert domain containing receptor)/flk1 (fms-like tyrosine kinase) expression on tumor cells (Chen et al., 2004a, b). VEGF has been shown to be a potent angiogenic factor. It binds to VEGF receptors present on the surface of endothelial cells and activates various functions of angiogenesis.

More recently, the antiangiogenic activity of ARTs has been shown to result in embryotoxicity (Li & Weina, 2010b). Although not yet reported to be a problem in clinical use, embryotoxicity has been reported for this compound class in both *in vitro* and *in vivo* experimental models, in particular after AS and/or DHA treatment, which resulted in embryo death and developmental abnormalities in early pregnancy in different animal species. The embryotoxicity appears to be connected with defective angiogenesis and vasculogenesis in certain stages of embryo development. DHA has been shown in rat whole embryo cultures to primarily affect primitive red blood cells causing subsequent tissue damage and dysmorphogenesis. AS has also been found to be embryolethal and teratogenic in rats, suggesting that embryonic erythroblasts are the primary target of AS toxicity in the rat embryo after *in vivo* treatment, preceding embryolethality and organ malformations (Medhi et al., 2009).

In particular, cancer angiogenesis plays a key role in the growth, invasion, and metastasis of tumors. Therefore, AS or DHA induced inhibition of angiogenesis could be a promising therapeutic strategy for treatment of cancer. Other anticancer mechanisms induced by AS and DHA have been recognized recently that have guided various clinical cancer trials. Given the fact that AS and DHA have been shown to inhibit vasculogenesis, these drugs should be avoided in pregnant patients with malaria or cancers in order to prevent fatal embryotoxicity. Further research on the mechanism of efficacy could lead us to understand more clearly the possibilities inherent in therapeutic development of ARTs for malaria, cancer, and other indications. Further study of the effects of ART compounds on vascularization and angiogenesis may improve the usage of AS and DHA for treatment of malaria and cancer. In this chapter, a thorough review of the antivascular mechanisms of AS/DHA therapy will be provided along with an extended discussion of the downstream embryotoxicity and anti-tumor effects of AS/DHA therapy.

2. Embryotoxicity of AS/DHA caused by defective vasculogenesis/hematopoiesis

Preclinical studies in rodents have demonstrated that ARTs, especially injectable AS, can induce fetal death and congenital malformations at a low dose. AS induced embryotoxicity can be shown in those animals only within a narrow window of time in early embryogenesis. Further evidence has been presented that the mechanism by which embryotoxicity of AS/DHA occurs seems to be limited to fetal erythropoiesis and vasculogenesis/angiogenesis in the very earliest developing red blood cells. The effect of

AS/DHA on red cell precursors has been shown to cause severe anemia in embryos with higher drug peak concentrations (Clark et al., 2004; White et al., 2008).

2.1 AS/DHA induces severe embryotoxicity in animal species

Artemisinin-based combination therapies (ACTs) and injectable AS are currently recommended by WHO (2006a) as frontline antimalarial treatments for uncomplicated and severe malaria, respectively, with over 100 million courses administered annually. While ART-based therapies have been shown to be well tolerated and efficacious, embryotoxicity has been reported in a number of animal species in a variety of research studies. In this chapter, we will thoroughly discuss all aspects of the various research studies that have been conducted on the embryotoxic mechanisms of ARTs in rodents and monkeys. Animal studies are very valuable in showing possible risks to humans from drug compounds, and a number of studies on reproductive risk associated with ARTs have been conducted showing the potential for embryotoxicity in the first trimester of pregnancy. In general, ART and its derivatives are considered safe and effective in pregnant women who have been treated with ART compounds, including a small number treated in the first trimester, the most vulnerable period for ART-induced embryotoxicity. In clinical trials, the patients did not show any increases in miscarriage or stillbirth with evidence of abnormality. A follow–up of exposed babies did not reveal any delays in child developmental (Efferth & Kaina 2010).

Studies in rodents have demonstrated that ARTs can induce fetal death and malformations at high oral doses and low injectable dose levels (Dellicour et al., 2007). Embryotoxicity can be induced by ARTs only within a narrow time window in rodents during early embryogenesis. Red cell progenitors are produced during a very limited time period during embryogenesis so that a single exposure to ARTs can result in a significant rise in hematopoietic cell death (WHO, 2006b; White et al., 2008). Treatment with AS/DHA has been shown to lead to a loss of hematopoietic precursors which may result in embryo death. In those embryos that survived, malformations were observed. Limited data in primates suggest that ARTs may have a similar mechanism of action in monkeys where AS/DHA treatment leads to anemia and embryolethality, however, the monkeys required more than 12 days of treatment to induce such embryonic death (Clark et al., 2008a). No embryo malformations were observed in the primate studies but these were limited in scope. The significant difference between the time periods where embryos were vulnerable to AS/DHA induced embryolethality in rodents and monkeys suggests that the time window for ART induced embryolethality is much longer for primates and may well be much longer for humans.

Preclinical studies of the reproductive toxicity of ARTs in various animal species showed that the injectable AS formulation has the greatest potential for embryotoxicity (Table 1). Following intravenous, intramuscular, and subcutaneous administrations, injectable AS was shown to have an drug dose induces 50% fetus re-absorbed (FRD_{50}), the dose required for 50% of the fetuses to be resorbed, of less than 1.0 mg/kg , which is much lower than the therapeutic dose of AS, which is 2–4 mg/kg in humans. In contrast, those animal species treated with oral ARTs and intramuscular artemether were shown to have a much higher FRD_{50} of 6.1–51.0 mg/kg (Table 1)(Li et al., 2010b). The mechanism for development of severe embryotoxicity in animals after treatment with injectable AS was not known in 2006, and research on this subject has only been published in recent years.

In later studies, more data on the mechanism of AS-induced embryotoxicity was shown. A study by Efferth and Kaina (2010) suggested that AS-induced embryotoxicity could be due to the antivasculogenic and antihematopoietic effects of AS/DHA on the embryonic

erythroblasts in the very earliest developing red blood cells. This study was inspired by anticancer studies conducted with AS (Efferth et al., 2001; 2006; Chen et al., 2003; 2004b).

Animal (drugs)	Dose duration	Dose regimens (daily)	Dosing route	No-observed-adverse-effect-level (NOAEL) on fetus resorption (mg/kg)				FRD_{100} (mg/kg)	FRD_{50} (95% CL) (mg/kg)
				Oral	IM	IV	SC		
Mice (AM)	GD 6-15	Multiple x 10	IM		5.4				11.3 (10.6 - 12.0)
(DHA)	GD 7	Single	SC				10		32.8 (27.7 – 38.9)
Rats (ART)	GD 1-6	Multiple x 6	Oral	5.6					11.5 (10.5 – 12.2)
(ART)	GD 7-13	Multiple x 7	Oral	7					≥ 35
(DHA)	GD 9.5, 10.5	Single	Oral	7.5					NA
(AM)	GD 6-15	Multiple x 10	Oral	2.5					14.4 (10.4 – 17.8)
(AS)	GD 6-17	Multiple x 12	Oral	5-7					7.74 (6.92 – 8.57)
(AS)	GD 10	Single	Oral					15.0	NA
(DHA)	GD 10	Single	Oral					11.1	NA
(AM)	GD10	Single	Oral					19.4	NA
(AE)	GD 10	Single	Oral					20.3	NA
(AS)	GD 11	Single	Oral					17.0	NA
(AS)	GD 6-15	Multiple x 10	SC				0.2		0.58 (0.55 – 0.61)*
(AM)	GD 6-15	Multiple x 10	IM		2.7				6.13 (4.52 – 8.26)
(AS)	GD 11	Single	IV			0.75		1.5	NA
(AS)	GD 6-13	Multiple x 13	IV, IM		0.5	0.4			0.60 - 0.61 (0.47-0.72)*
Hamster (DHA)	GD 7	Single	SC				4.2		6.06 (5.90 – 6.21)
(DHA)	GD 7	Single	Oral	20					51.0 (37.9 – 68.7)
(AS)	GD 5	Single	SC				0.35		1.0 (0.9 – 1.2)*
Guinea Pig (DHA)	GD 18	Single	IM		2.5				18.3 (13.9 – 24.2)
Rabbits (AM)	GD 7-18	Multiple x 12	IM		0.7				NA
(AS)	GD 7-19	Multiple x 13	Oral	5-7					NA
(DHA)	GD 9	Single	IM		5.0				7.57 (7.48 – 7.67)
Monkey (AS)	GD 20-50	Multiple x 12	Oral	4					≥ 12

FRD_{50} or $_{100}$ = drug dose induces 50% or 100% fetus re-absorbed; GD = gestation day
(The day of mating was defined as day 0 of gestation.)
* The severe toxic effects were detected in the animals treated with AS after single or multiple intramuscular, intravenous or subcutaneous injections. Values of ED_{50} are given as median (95% confidence limits). NA = not available.

Table 1. Embryotoxic effects (NOAEL and $FRD_{50 \text{ or } 100}$) of artemisinin (ART), dihydroartemisinin (DHA), artesunate (AS), artemether (AM) and arteether (AE) given intragastrically (Oral), intravenously (IV), intramuscularly (IM), and subcutaneously (SC) in pregnant mice, rats, hamster, guinea pig, rabbits, and monkeys (Li & Weina, 2010b).

2.2 AS/DHA therapy causes erythrocyte depletion and resulting embryotoxicity

The antihematopoietic, antivasculogenic, and antiangiogenic effects of artemisinins (ARTs) have been shown through *in vitro* and *in vivo* studies, which have been summarized in Table 2. This comprehensive list of publications provides a reference list of current work on AS-induced embryotoxicity.

Drugs	*in vitro* studies	Drugs	*in vivo* studies
ART	Increased levels of ROS and inhibition of angiogenesis in mouse embryonic stem cell-derived embryos	AS/DHA	Death and loss of primitive erythroblasts, reduction of maternal reticulocyte count, and anemia in embryos of rodents
ART	Down regulation of VEGF and HIF-1a in mouse embryonic cells, impairment of laminin organization, and impaired expression of MMP-1, 2, and 9.	AS	Embryonic death and fetal resorption during organogenesis in rats and monkeys
DHA	DHA effects on primitive RBCs lead to anemia, cell damage, and high concentrations of DHA inhabited vasculogenesis and angiogenesis in rodents	AS/AM/AE	Retardation of fetal growth among surviving fetuses, cardiotoxicity, heart and skeletal defects, delays in limb and tail development in rats
DHA	Reduction of primitive red blood cells in frog embryos	AS/AM/AE	Significant necrosis of embryonic livers in rats

AS = artesunate; DHA = dihydroART; ART = ART; ROS = reactive oxygen species; VEGF = vascular endothelial growth factor; HIF-1a = hypoxia-inducible factor 1a; MMP = matrix metalloproteinase;

Table 2. A summary of the research findings and possible mechanisms of artemisinin drugs in the embryotoxicity studies *in vitro* and *in vivo* (Clark et al., 2009; Efferth & Kaina 2010; Finaurini et al., 2010; Longo et al., 2006a, 2006b, 2008; Wartenberg et al., 2003; White et al., 2008)

2.2.1 Inhibitory effects of AS/DHA on hematopoiesis

AS is embryolethal and teratogenic in rats, with a very narrow window of sensitivity between days 10-14 of gestation (Clark et al., 2004; Li et al., 2009; White et al., 2006). Further studies with a single oral dose of 17 mg/kg AS on gestational day (GD) 10-11 demonstrated that a paling of the visceral yolk sacs was observed within 3-6 hr after treatment. Within 24 hr, marked paling was observed which persisted through GD 14. Histologically, embryonic erythroblasts were reduced; cells showed signs of necrosis within 24 hr, and the erythroblasts were maximally depleted by 48 hr (White et al., 2006). The depletion of embryonic erythroblasts was shown to be the root cause of prolonged and severe anemia in AS-induced developmental toxicity observed *in vitro* using whole embryo rat cultures (WEC) (Longo et al., 2006a).

To verify the primary target of DHA in WEC and to detect consequences induced by early damage on embryo development, pregnant female rats were orally treated on GD 9.5 and 10.5 with 7.5 or 15 mg/kg/day DHA. A parallel *in vitro* WEC study evaluated the role of oxidative damage and examined blood islands and primitive RBCs. Embryos were observed for yolk sac hematopoiesis (Wolffian blood island formation, vasculogenesis and hematopoiesis) at stages known to be affected by DHA exposure. In accordance with the WEC results, primitive RBCs from yolk sac hematopoiesis were shown to be the target of DHA *in vivo*. The resulting anemia led to cell damage, which depending on its degree, was either diffuse or focal. Embryonic response to acute anemia varied from complete recovery to malformation and death. The malformations were shown to occur only in litters with embryonic deaths. DHA has been shown to induce low glutathione levels in RBCs,

indicating that oxidative stress may be involved in ART toxicity. These effects were shown to be extremely rapid, with altered RBCs seen as early as GD 10.

In early development, the first wave of embryonic erythropoiesis begins in the visceral yolk sac on GD 7–7.5 in the mouse (Baumann & Dragon, 2005; Palis & Yoder, 2001) corresponding to GD 8.5–9 in the rat. Clusters of progenitor cells (hemangioblasts) form blood islands and differentiate into primitive erythroblasts and vascular endothelial cells (Tavassoli, 1991). The primitive erythroblasts enter the embryonic circulation on GD 10 in the rat, at about the same time that the heart begins to beat. These are a self-sustaining population of nucleated cells that proliferate while in the circulating blood. The definitive embryonic erythroblasts begin entering the circulation at about GD 13 in the rat. They are formed in the liver from hematopoietic stem cells which originate in the visceral yolk sac and migrate to the liver (Palis and Yoder, 2001; Tavassoli, 1991). The marked depletion of embryonic erythroblasts after a single AS dose to rats on GD 10 or 11 (White et al., 2006) is due to the killing of the primitive erythroblast population. As the primitive erythroblast population is self-sustaining after GD 10, this population cannot be replaced after extensive depletion until definitive erythroblasts are introduced into the blood from the liver.

During the embryotoxicity of AS, the replacement of these erythroblasts may fail because the embryonic liver has also been shown to be a target for AS in rats. Abnormalities in liver shape and lobation were caused by all four ARTs tested (AS, DHA, artemether and arteether) when administered as a single dose on GD 10 (White et al., 2008; Clark et al., 2008b). Fetal liver necrosis was observed in this study, which could be the basis for the unusual liver shape variation that we observed in this study of the narrow window of AS embryotoxicity. Histologically, the livers of these animals showed marked necrosis involving both hepatocytes and developing hematopoietic cells on GD 13 following a single dose of 17 mg/kg AS on GD 10.5 or 11 (White et al., 2006). In addition, the hematopoietic cells in the adult and embryo have been shown to be the target for selective binding of radiolabeled AS (Clark, 2009).

2.2.2 Relevance of vasculogenesis and hematopoiesis to embryo development

In the vertebrate embryo, hematopoietic and vascular endothelial cells are the first cells in the visceral yolk sac to differentiate in response to induction of the mesoderm (Baron 2001). A common precursor cell, the hemangioblast, gives rise to both erythrocytes and endothelial cells within blood islands. Yolk sac or primitive hematopoiesis is restricted to the formation of nucleated erythrocytes that express embryonic hemoglobin (Wong et al., 1986) and macrophages (Cline & Moore, 1972). At about 12 days of gestation this extra-embryonic hematopoiesis gives way to intraembryonic "definitive" hematopoiesis, first in the fetal liver and later the bone marrow (BM) and spleen. Definitive hematopoiesis is the process whereby all types of blood cells are formed followed by their differentiation, including the enucleation of erythrocytes.

As embryonic development continues, the site for hematopoiesis changes to the fetal liver, and, as the animal matures, to the bone marrow, where hematopoiesis persists in the adult. During development of the yolk sac, hematopoiesis is intimately linked to the development of blood vessels. In fact, the ontogenic relationship between hematopoietic cells and the endothelial cells of the blood islands of the yolk sac was confirmed many years ago (Sabin, 1917). These observations suggest that vasculogenesis and hematopoiesis are part of the same process, in which formation of a blood vessel is accompanied by the simultaneous in situ production of blood cells within that vessel, a process known as hemo-vasculogenesis

(Sequeira Lopez et al., 2003). In this environment, the first hematopoietic structure is responsible for the production of the primitive erythrocytes and the first transient population of definitive erythrocytes. In parallel, angioblasts multiply and differentiate into endothelial cells. The endothelial cells form tubes and then a network of capillaries that eventually reaches the circulation of the embryo (Baumann & Dragon, 2005).

This widespread phenomenon occurs by hemovasculogenesis, the formation of blood vessels accompanied by the simultaneous generation of red blood cells. Erythroblasts develop within aggregates of endothelial cell precursors. When the lumen forms, the erythroblasts "bud" from endothelial cells into the forming vessel. The extensive hematopoietic capacity found in the embryo helps explain why, under pathological circumstances such as severe anemia, extramedullary hematopoiesis can occur in any adult tissue. Understanding the intrinsic ability of tissues to manufacture their own blood cells and vessels has the potential to advance the fields of organogenesis, regeneration, and tissue engineering (Sequeira Lopez et al., 2003).

Previous data confirm the rapid onset of action of DHA on primitive RBCs, as soon as they enter circulation. There is no clear explanation why primitive RBC's are susceptible to DHA (Longo et al., 2006a and 2006b). Like intraerythrocytic malaria parasites, primitive RBCs have high concentrations of iron and heme, which have been proposed as either activators or targets of ART compounds (Olliaro et al., 2001; Parapini et al., 2004). The depletion of embryonic erythroid cells is believed to occur as a consequence of AS/DHA inhibition of vasculogenesis. This hypothesis is supported by the fact that vasculogenesis and hematopoiesis are part of the same process, in which formation of a blood vessel is accompanied by the simultaneous in situ production of blood cells within that vessel (Baron, 2001; Baumann & Dragon, 2005; Sequeira Lopez et al., 2003). In addition, oral and injectable AS treatment has been shown to induce marked embryolethality and a low incidence of teratogenic effects, including cardiovascular defects (ventricular septal and great vessels defects), which significantly affected novel vessel formation (Clark et al., 2004, White et al., 2008; Ratajska et al., 2006a, 2006b).

2.2.3 Antivasculogenic and antiangiogenic effects of AS/DHA

Red blood cells and the cardiovascular system are the first embryonic organ systems to show overt differentiation. Together they constitute the principal support system of the growing embryo (Baumann & Dragon, 2005). Vessel development within the embryo occurs via two processes: vasculogenesis and angiogenesis (Rongish et al., 1994; Risau, 1995, 1997). Vasculogenesis is the formation in situ of coronary vessels from endothelial cell progenitors (angioblasts) or angioblast migration to areas of vessel formation and their subsequent differentiation into vascular channels. Angiogenesis is the development of vessels from preexisting ones by capillary sprouting, intussusceptive growth, and remodeling (Risau, 1997; Ratajska et al., 2006a, 2006b). The first morphological signs of vasculogenesis within an embryo are "blood islands," which consist of erythroblasts and premature endothelial cells (angioblasts). Blood islands are encountered within the yolk sac of GD 7.5–13 rat embryos. The first signs of vasculogenesis in heart development are blood island-like structures.

In a series of AS/DHA embryotoxicity studies, heart abnormalities (swollen or collapsed chambers) were observed within 24 hr in 25–60% of embryos and within 48 hr in 100% of embryos, correlating with histological signs of cardiac myopathy (thinned and underdeveloped heart walls and enlarged chambers) (White et al., 2006). Rats deficient for

these genes had normal heart development through GD 12.5–13, dilated hearts with reduced trabeculation, histological evidence of a thinned compact zone, and embryonic death occurred by GD 11. In light of this information, White's data (2008) are consistent with the hypothesis that depletion of embryonic erythroblasts creates a hypoxic environment, limiting the availability of oxygen as a substrate for oxidative phosphorylation in the heart. As a result, the energy requirements for normal proliferation of heart tissue are not met, causing underdevelopment of the heart. This could then lead to impaired cardiac function and hemodynamic changes, resulting in ventricular septal defects and great vessel malformations (Clark et al., 2004), which could further impair cardiac function. The hypoxic conditions created by both anemia and impaired cardiac function could then lead to embryonic death (White et al., 2008).

New patterns of malformations not seen previously were observed in studies in which AS was administered orally at closely spaced doses during organogenesis in pregnant rats and rabbits (Clark et al., 2004). The malformations consisted of cardiovascular malformations (ventricular septal defects and various vessel defects) and a syndrome of skeletal defects including shortened and/or bent long bones and scapulae, misshapen ribs, cleft sternebrae, and incompletely ossified pelvic bones. In rats, malformations occurred only at embryolethal doses. The embryolethal effect occurred with a steep dose-response curve and was apparent as abortions in rabbits and post-implantation loss including total litter loss in both rats and rabbits. These developmental effects were observed largely in the absence of any apparent maternal toxicity (Clark et al., 2009).

Compared to rat embryos, the larvae of frogs treated with AS/DHA had no areas of necrosis but they shared similar heart defects. Heart defects were seen in both frog species treated with doses of DHA ≥ 0.1 μg/mL (abnormal heart looping, thin atrial and ventricular walls and underdeveloped trabeculae). The fact that cardiac defects are seen in Xenopus larvae in the absence of cell death offers an alternative explanation for the mode of action of ART. In rats these findings were attributed to cell death resulting from anemia and hypoxia (Longo et al., 2006a); here, frog larvae death may be secondary to anemia. Potentially larvae death may not be mediated by cell death but rather by altered intracardiac hemodynamics due to RBC degeneration, as speculated by White et al. (2006).

In embryotoxicity studies conducted with DHA administered at GD 9, the yolk sac was shown to be fully vascularized. However, at DHA concentrations ≥ 0.05 μg/mL, the visceral yolk sac was well vascularized but pale; yolk sac vessel formation and circulation appeared normal, but blood in the yolk sac was visibly paler than blood observed in the yolk sacs of control animals. This effect increased with higher DHA doses. At concentrations ≥ 0.5 μg/mL, the vasculature was also affected. Vessel diameter was reduced, small anastomotic vessels were not visible, and the number of circulating cells was further reduced. At DHA concentration of 1.0 μg/mL, yolk sac vasculature and circulating cells were markedly reduced, and at 2 μg/mL only a few poorly organized yolk sac vessels were present. At DHA concentrations ≥ 2 μg/mL, the heart-beat of the majority of the embryos was reduced and irregular.

These observations showed higher concentration and longer time exposure of AS/DHA inhibited vasculogenesis and angiogenesis (Longo et al., 2006a; 2006b; White et al., 2008). Therefore, the embryonic heart defects and killing of embryonic primitive erythroblasts after AS/DHA treatment significantly affected blood vessel development and the formation of a circulatory system. The sequence of defects noted began with the loss of primitive erythroblasts and ended with inhibition of heart vascularization (Finaurini et al., 2010; Kwee et al., 1995; Ratajska et al., 2006a; 2006b; Sequeira Lopez et al., 2003).

2.3 Possible antivascular mechanism associated with artemesinin-induced embryotoxicity

Current hypotheses on the pharmacological and toxicological mechanisms of ART action propose that the endoperoxide group of the ARTs functions as both the pharmacophore and toxicophore, and that chemical activation of these compounds is essential for activity. The antimalarial mode of action of the ARTs is thought to be related to activation of the endoperoxide bridge by heme-derived or chelatable iron during the erythrocytic stage of malaria, producing centered radicals via a well-characterized chemical pathway. Ultimately, it is these radicals that are the toxic species. The peroxide bridge is activated via the association of iron, in the form of Fe^{2+}, to either oxygen of the peroxide group, generating two distinct oxyl radicals (Mercer, 2009).

Evidence for the role of the mitochondria in both the pharmacological and toxicological mechanisms of action of the ARTs has been reported. The mitochondrion has long been recognized as a site of endoperoxide accumulation within plasmodium parasites, and this accumulation is co-incident with observed mitochondria damage (Maeno et al., 1993). Studies have also demonstrated that ARTs bind to active, not inactive, mitochondria in rat embryo erythroblasts (White et al., 2006; 2008). Other studies have further implicated mitochondria in the pharmacological and toxicological mechanism of action of ARTs. For example, researchers studying the ART mechanism of action using a yeast system published data to propose that the mitochondria play a dual role: the electron transport chain stimulates the effect of ART, most probably via activation of the peroxide group, and the mitochondria are subsequently damaged by locally generated free radicals (Li et al., 2005).

A recent study demonstrated that mitochondria are the main subcellular target of endoperoxides in primitive red blood cells in the Xenopus frog embryo teratogenesis model system (Longo et al., 2008). This study suggests that the mitochondria are a site of activation for DHA. This is an attractive premise as it can be used to explain the selectivity of AS/DHA between quiescent cells and rapidly proliferating cancer cells. In dividing cells, mitochondria are active in order to deliver sustained energy for continued growth. Furthermore, the presence of a high number of iron-sulfur containing enzymes, the redox activity of the electron transport chain and its role in the initiation of apoptosis are compatible with the hypothesized cytotoxic action of the ARTs via a one-electron reduction of the peroxide bridge to cytotoxic radical species. In addition, studies have demonstrated that endoperoxide activity is accompanied by the generation of oxidative stress, which is often associated with mitochondrial dysfunction (Disbrow et al., 2005). This rationale supports the role of mitochondria as a possible activating system and/or target of the ARTs and opens up an exciting new area for research in establishing the role of bioactivation and mechanism of action (Mercer, 2009).

2.4 Circumventing embryotoxicity in pregnant women treated with AS/DHA

Embryotoxicity, toxicokinetics, and tissue distribution studies after intravenous and intramuscular AS treatment of pregnant and non-pregnant animals have also been conducted (Li et al, 2008 & 2009). These data demonstrate that the severe embryotoxicity induced by injectable AS is due to a number of factors: 1) injectable AS treatment results in a much higher peak drug concentration than treatment with oral ARTs and intramuscular artemether; 2) DHA produced by hydrolysis of AS plays a key role in embryotoxicity; 3) Among all ARTs, AS has the highest conversion rate to DHA; 4) the conversion rate of AS to DHA has been shown to be significantly increased in pregnant animals; 5) the buildup of high peak concentrations of AS

and DHA in the blood of pregnant rats has been shown to be significantly higher than those of non–pregnant animals; and 6) injectable AS treatment results in higher distribution of AS and DHA in feto–placental tissues in pregnant animals (Li et al., 2008).

2.4.1 Toxicity of AS/DHA occurs in a narrow time window during embryogenesis

Studies in rodents have demonstrated that ARTs can induce fetal death and congenital malformations at low dose levels, which can be induced in rodents only within a narrow window in early embryogenesis (GD 10-14). A recent study in cynomolgus monkeys found that AS treatment caused embryo death between GD 30 and 40. The no-observed-adverse-effect-level (NOAEL) was 4 mg/kg/day. No malformations were observed in four surviving fetuses in the 12 mg/kg/day group, but the sample size of this study was not adequate to conclude that AS is not teratogenic at that dose in monkeys. All three live embryos in the 30 mg/kg/day AS group dosed from GD 20 were removed by caesarean section on GD 26, 32 and 36 respectively, and these embryos showed marked reduction in erythroblasts. Since embryo death was observed only after more than 12 days of daily treatment at 12 mg/kg, the lack of developmental toxicity at a dose of 30 mg/kg/day indicates that a shorter treatment period decreases the potential for AS induced embryotoxicity in monkeys (Clark et al., 2008a).

Primitive erythroblasts develop in the visceral yolk sac and are released into the embryonic circulation on GD 10 in rats, at about the same time that the heart begins to beat. If the primitive erythroblasts are also the primary target of AS action in monkeys, then the most sensitive time window for embryotoxicity would be when those primitive erythroblasts predominate in the embryonic circulation. In the cynomolgus monkey, the heart starts beating at about GD 18. Although no data exist at this time to prove the timing of the switchover from primitive to definitive erythroblasts in monkeys, the erythroblasts visible in the sections of embryos from GD 26, 32, and 36 were > 90% nucleated, suggesting that they were probably primitive erythroblasts during GD 18 to 36. On GD 50, only 9% of blood cells were nucleated, indicating that the transition from primitive to definitive erythroblasts was nearly complete on GD 50 (Clark et al., 2008a).

The time window of AS/DHA sensitivity observed in animal studies would hypothetically correspond to humans during organogenesis. The earliest primitive erythrocytes are formed in the yolk sac starting at GD 18.5 (Clark et al., 2008a; Lensch & Daley, 2004). The onset of blood circulation coincides with the onset of the embryonic heartbeat, which probably occurs between GD 19 and GD 21 in humans, evidenced by the appearance of primitive erythrocytes in the cardiac cavity. The liver is the first organ to be colonized by the yolk sac and is the main site of definitive erythropoiesis from week 5 through week 24 of gestation (Segel & Palis, 2001). Primitive erythrocytes are the predominant circulating form in the first 8 to 10 weeks of gestation. Liver–derived definitive erythrocytes begin to enter the circulation by 8 weeks of gestation, but do not predominate until 11 to 12 weeks (Kelemen et al., 1979). All available studies agree that yolk sac hematopoiesis disappears completely after GD 60 (Lensch & Daley, 2004).

Therefore, the timing of the switchover from primitive to definitive erythroblasts is GD10–14 for rats, GD 18–50 for monkeys, and GD 16–60 for humans. Based on this information, if human embryos were sensitive to AS or DHA in the same way as rat and monkey embryos, then the most sensitive period for human development toxicity induced by ARTs would be predicted to begin with the onset of circulation in week 4 of gestation and end at approximately week 9 to 10 of gestation. At this time in gestation, the nucleated primitive

erythroblasts have been largely replaced by non–nucleated definitive erythroblasts (Clark et al., 2008a). If primitive erythrocytes are formed over a longer period than that in rodents, then AS/DHA dosing for longer than 12 days may be required to produce a severe effect on the early blood cell population in primates or humans (Clark et al, 2008;WHO2006b).

2.4.2 Pharmacokinetic concerns of AS/DHA relating to embryotoxicity

Although no animal species exists that completely mimics man, non-human primates provide the best comparison to humans. With animal experiments only certain aspects of a highly complex system can be analyzed. In order to achieve this successfully, animal species and experimental set–up have to be chosen carefully to represent the human condition in as suitable a model as possible. The more the model deviates from the human condition the less predictive it will be. Today, more information is available on the pharmacokinetic and toxicokinetic properties of ARTs (Li & Weina, 2010b). This will help provide data on the embryotoxic/teratogenic doses of a substance or on their non–embryotoxic/teratogenic doses relevant to man. In addition, the relative duration of exposure to three day ACT for malaria in humans, with respect to the duration of organogenesis, may be too short to induce the severe embryotoxicity that is observed in animal species.

Another consideration when reviewing pharmacokinetic implications of AS/DHA therapy is the impact of pregnancy on drug metabolism. There are physiological changes in pregnancy that can cause a decrease in plasma drug concentrations and the area under the curve (AUCs), resulting in reduced efficacy (Dawes & Chowjenczyk, 2001). This is likely due to increased clearance, larger volume of distribution and perhaps altered absorption following oral administration. Clearly, oral dosages of these antimalarial drugs need to be adapted to maintain efficacy when given to pregnant patients and animals with malaria (Nosten et al., 2006). However, data obtained for the pharmacokinetic parameters for these antimalarial agents show no significant difference between pregnant and non–pregnant women and animal species after single intravenous or intramuscular injections (Li et al., 2008; Menendez 2006; Newton et al., 2000).

Preclinical studies of AS-induced embryotoxicity in pregnant rats has shown thta injectable AS administration resulted in severe toxicity to animals by routes of intraveous, intramuscular, and subcutaneous administration with doses higher than 1.0 mg/kg. However, animals treated with oral ARTs and intramuscular AM demonstrated that these ARTs were much safer than injectable AS with embryolethality demonstrated at doses ranging from 6.1–51.0 mg/kg (Table 1). Similar findings showed that treatment with a single dose of 17 mg/kg oral AS and 1.5 mg/kg intravenous AS administered on GD 11 both caused 100% embryolethality and are close to the threshold for that effect (10 mg/kg oral AS caused only 15% fetal resorption and 0.75 mg/kg intravenous AS caused 7%fetal resorption) (Clark et al., 2009; White et al., 2008). Toxicokinetic and tissue distribution data demonstrated that the severe embryotoxicity induced by injectable AS is due to the following six factors (Li et al., 2008).

1. Injectable AS can provide much higher peak concentrations (3–25 fold higher) than oral ARTs and intramuscular AM in animals (Li et al., 2008). *In vitro* results in other studies have shown that the drug exposure level and time are important to induce embryotoxicity (Longo et al., 2006a; 2006b; 2008). However, *in vivo* studies demonstrated that the drug exposure level is more important than drug exposure time because AS and DHA have very short half–lives (≤ 1 h) in animal species (Li et al., 2008).

2. AS is completely converted to DHA and is basically a prodrug of DHA. Also, DHA was shown to be more effective than AS in inhibition of angiogenesis and vasculogenesis *in vitro* (Longo et al., 2006a; 2006b; Chen et al., 2004b; White et al., 2006). In addition without DHA formation, the embryotoxicity of ARTs can be reduced by using artemisone, which has significantly less anti–angiogenic activity than DHA. Artemisone is a novel derivative of ART and is not metabolized to DHA (D'Alessandro et al., 2007; Schmuck et al., 2009).
3. The conversion rate of AS to DHA is the highest among all ARTs. The conversion rate of AS to DHA was shown to be 38.2–72.7% in comparison to that of AM and AE which show a conversion rate of 12.4–14.2% in animal species (Li et al., 2008).
4. In contrast to the data observed with single AS dosing, the conversion rate of AS to DHA was significantly increased in pregnant rats compared to non–pregnant animals following multiple injections. The concentrations of DHA generated in pregnant rats was 2.2–fold higher on day 1 and 4.5–fold higher on day 3 than that observed in non–pregnant animals, resulting in a total AUC_{D1-3} of 15,049 ng·h/ml which is 3.7–fold higher in pregnant rats than the AUC_{D1-3} of 4,015 ng·h/ml observed in non–pregnant rats. The ratios of AUC_{DHA}/AUC_{AS} were also shown to be 0.99–1.02 for pregnant rats and 0.42–0.48 for non–pregnant animals, indicating that the total exposure of pregnant rats to DHA during the whole period of treatment was much higher than in non–pregnant rats (Li et al., 2008).
5. The buildup of high peak concentrations of AS and DHA in the plasma of pregnant rats was significantly higher than those of non–pregnant animals after repeated dosing. In a study of the toxicokinetics (TK) of AS, the data revealed that the peak concentration (C_{max}) of AS (14,927-16,545 ng/ml) in pregnant rats was double the C_{max} of AS (5,037-8,668 ng/ml) in non–pregnant animals. Comparable to the C_{max} values, the mean AUC data of DHA showed values much higher in pregnant animals (3,681–4,821 ng·h/ml) than those observed in non–pregnant rats (1,049-1,636 ng·h/ml). The TK results also showed the mean AUC of DHA was significantly increased from day 1 (3,681 ng·h/ml) to 3 (4,821 ng·h/ml) in the pregnant rats, but remarkably decreased from day 1 (1,636 ng·h/ml) to Day 3 (1,049 ng·h/ml) in the non–pregnant animals (Li et al., 2008).
6. Injectable AS can also result in higher distribution of AS and DHA in the tissues of feto–placental tissues in pregnant animals after multiple administrations. A tissue distribution study of ^{14}C–AS showed that the total AUC_{0-192h} of the radioactive labeled AS was 22,879 µg equivalents·h/g in all measured tissues of the pregnant rats, and 6.54% of the total radioactivity was present in all of the feto–placental tissues. During the 192 h treatment period, measured levels of radioactivity in the ovary, placenta, and uterus were 555, 367, and 216 µg equivalents·h/g, respectively. The values observed in feto-placental tissues were more than 2–4 fold higher than observed in blood (134 µg equivalents·h/ml) (Li et al., 2008).

The observed pharmacokinetics of antimalarials is altered in pregnancy after oral administration, and the drug plasma levels are decreased. However, previous data has shown that AS and DHA concentrations in the plasma and reproductive tissues of pregnant rats were significantly increased over the AS and DHA concentrations observed in non–pregnant animals after injectable AS treatment. The significant increase in blood ant tissue concentrations of AS and DHA may be related to the severe embryotoxicity observed after treatment of pregnant animals with injectable AS even in low dose regimens.

2.4.3 Current safety regimens for artemisinin therapy in pregnant women

There are three facts that support the assertion that oral ARTs are safe in pregnant women: 1) the pharmacokinetics of antimalarials is altered in pregnancy after oral administration, which can cause a decrease in drug exposure levels; 2) treatment with oral ARTs has been shown to result in low peak concentrations when compared to the peak concentrations observed after treatment with injectable ARTs as discussed above; and 3) data on clinical trials regarding the possible effects of ARTs on pregnancy have not shown any embryotoxic effects in humans for the past 25 years with oral ART monotherapy or oral ACTs.

Few studies in pregnant women have been published for ARTs or other antimalarials. For example, oral chloroquine prophylaxis treatment, oral mefloquine therapy, oral progaunil treatment as well as oral DHA therapy all show altered kinetics in pregnancy, and plasma levels of these drugs are significantly lower than those observed in non-pregnant patients with malaria (Li & Weina, 2010b). This is likely due to increased clearance, larger volume of distribution and perhaps altered absorption following oral administration. In comparison to non–pregnant Thai women, the C_{max} and AUC of DHA values were 4.2 and 1.8 fold, lower in pregnant patients (McGready et al., 2006; Ward et al., 2007). A similar observation has also been found in animal studies of oral administration of AS (Clark et al., 2004). Clearly, oral dosage of these antimalarials needs to be adapted to maintain efficacy when given to pregnant patients and animals with malaria (Nosten et al., 2006). In this case, treatment with oral drugs appears safe due to the decreased drug exposure and fast elimination in pregnant women.

There is now a reasonable body of evidence for safety from most of the clinical trials published from 1989 to 2009 in nearly 1,837 pregnant women exposed to an ART agent or ACT with 176 pregnant patients in the first trimester. There were no clinically significant adverse effects of the drug treatment, nor any adverse outcome of the pregnancies, nor any adverse outcomes related to development (neurological and physical) of the infants, including 44 infants exposed during the first trimester (Li & Weina 2010b). Recent data published by the WHO (2006b) provided data on ART exposure in pregnancy from ongoing studies in Thailand, Zambia and Bangladesh. In Thailand, a study was recently conducted on 1,530 first trimester exposures to a range of antimalarial medicines including 170 pregnant women treated with ARTs. The highest risk of abortion in pregnant women with *P. falciparum* malaria treated with any antimalarial was associated with the number of episodes of infection and the number of times the women had to be treated in the first trimester. In addition, fever, hyperparasitemia and older maternal age were significant positive risk factors for an abortion in the first trimester, whereas antimalarial drug treatments were not significantly related.

The WHO concluded that there is insufficient evidence at present to warrant a change in the current WHO policy recommendations on the use of ACTs for the treatment of malaria in pregnancy. Current WHO Guidelines recommend that in uncomplicated malaria, ACTs should be used in the second and third trimester, but ACTs should only be used in the first trimester if they are the only effective treatments available (WHO 2006a). These recommendations are still valid based on the data presented in this chapter. However, the medicine of choice for initial treatment in the first trimester of pregnancy varies because of differences in drug sensitivities in different regions. The immediate use of ARTs is justified in situations where the first treatment fails because of the dangers of repeated malaria infections during later pregnancy. In the future, ACTs may be used to treat pregnant women in all trimesters after review of further safety studies to evaluate the risk of embryotoxicity.

3. Anticancer effect of artemisinins (ARTs) via an antiangiogenic mechanism

ART and its bioactive derivatives (AS, DHA, artemether and arteether) exhibit potent anticancer effects in a variety of human cancer cell model systems (Figure 1). The pleitropic response in cancer cells includes: 1) growth inhibition by cell cycle arrest, 2) apoptosis, 3) inhibition of angiogenesis, 4) disruption of cell migration, and 5) modulation of nuclear receptor responsiveness. These effects of ARTs result from perturbations of many cellular signaling pathways.

3.1 *In vitro* and *in vivo* research on anticancer effects of ARTs

3.1.1 Anticancer properties of ARTs

Molecular, cellular and physiological studies have demonstrated that, depending on the tissue type and experimental system, ART and its derivatives arrest cell growth, induce an apoptotic response, alter hormone responsive properties and/or inhibit angiogenesis of human cancer cells. The Developmental Therapeutics Program of the National Cancer Institute (NCI), USA, which analyzed the activity of AS on 55 human cancer cell lines (IC_{50} values shown between 0.512 and 124.295 mM, depending on the cancer cell line), showed that AS has strong anticancer activity against leukemia and colon cancer cell lines, and has intermediate effects on melanomas, breast, ovarian, prostate, central nervous system, and renal cancer cell lines (Efferth et al., 2001, 2006).

Moreover, the highly stable ARTs and ART-derived trioxane dimers were shown to inhibit growth and selectively kill several human cancer cell lines without inducing cytotoxic effects on normal neighboring cells. One proposed mechanism by which ART targets cancer cells is cleavage of the endoperoxide bridge by the relatively high concentrations of iron in cancer cells, resulting in generation of free radicals such as reactive oxygen species (ROS) and subsequent oxidative damage as well as iron depletion in the cells. This mechanism resembles the action of ART in malarial parasites. In addition to possessing higher iron influx via transferrin receptors, cancer cells are also sensitive to oxygen radicals because of a relative deficiency in antioxidant enzymes. A significant positive correlation can be made between AS sensitivity and transferrin receptor levels as well as between AS sensitivity and expression of ATP binding cassette transporter 6.

Expression profiling of several classes of tumor cells revealed that ART treatment caused selective expression changes of many more oncogenes and tumor suppressor genes than genes responsible for iron metabolism, which suggests that the anticancer properties of ARTs cannot be explained simply by the global toxic effects of oxidative damage. ARTs have also been observed to attenuate multidrug resistance in cancer patients, an effect due in part to the inhibition of glutathione S-transferase activity. ART and its bioactive derivatives elicit their anticancer effects by concurrently activating, inhibiting and/or attenuating multiple complementary cell signaling pathways, which have been described in a variety of human cancer cell systems as well as in athymic mouse xenograft models. The ART compounds exert common as well as distinct cellular effects depending on the phenotype and tissue origin of the examined human cancer cells. (Firestone & Sundar 2009)

3.1.2 *In vitro* anticancer effect of ART and its derivatives

While most of the investigations on the anticancer activities of ARTs have been performed with cell lines *in vitro*, there are a few reports in the literature showing activity *in vivo* against xenograft tumors, e.g., breast tumors, ovarian cancer, Kaposi sarcoma, fibrosarcoma,

or liver cancer. The *in vitro* data in the literature supports the hypothesis that ART derivatives kill or inhibit the growth of many types of cancer cell lines, including drug-resistant cell lines, suggesting that ART could become the basis of a new class of anticancer drugs. In addition, the co-administration of holotransferrin and other iron sources with ARTs has been shown to increase the potency of ARTs in killing cancer cells.

Artemisinin (ART)

ART was tested using a drug-sensitive H69 human small-cell lung carcinoma (SCLC) cell line and also by using multi-drug-resistant (H69VP) SCLC cells pretreated with holotransferrin. The cytotoxicity of ART on H69VP cells (IC_{50} = 24 nM) was ten-fold lower than for H69 cells (IC_{50} = 2.3 nM). Pretreatment with 880 nM holotransferrin did not alter the cytotoxicity of ART on H69 cells, but significantly enhanced the effect on H69VP cells (IC_{50} = 5.4 nM) (Sadava et al., 2002).

A recent study demonstrated that ART induced cell growth arrest in A375M malignant melanoma tumor cells, and also affected the viability of A375P cutaneous melanoma tumor cells with both cytotoxic and growth inhibitory effects, while ART was not effective in inhibiting growth of other tumor cell lines (MCF7 and MKN). In addition, ART affected the migratory ability of A375M cells by reducing metalloproteinase 2 (MMP-2) productions and down-regulating αvβ3 integrin expression. These findings introduce a potential of ART as a chemotherapeutic agent in melanoma treatment (Buommino et al., 2009).

Dihydroartemisinin (DHA)

DHA selectively killed Molt-4 lymphoblastoid cells when co-incubated with holotransferrin, while the same treatment was significantly less toxic to normal human lymphocytes. The drug combination of DHA and holotransferrin was approximately 100 times more effective on Molt-4 cells than normal lymphocytes (LC_{50s} of Molt-4 and normal lymphocytes were 2.6 μM and 230 μM, respectively). Incubation with DHA alone was found to be less effective than in combination with holotransferrin, indicating that intracellular iron plays a role in the cytotoxic effect (Lai & Singh, 1995).

HTB 27 cells, a radiation-resistant human breast cancer cell line, were killed effectively (reduced to 2% of original concentration) after 16 hr of treatment with DHA (200 μM) and holotransferrin (12 μM). However HTB 125 cells, a normal breast cell line, were not significantly affected by the same treatment. Also, when breast cancer cells were treated with only DHA (200 μM) (without holotransferrin), the cytotoxicity observed was significantly lower (Singh & Lai, 2001).

DHA is also cytotoxic to human glioma cells (U373MG), and the cytotoxicity is markedly enhanced by the addition of holotransferrin (Kim et al., 2006). In addition, radiation-induced expression of the endogenous antioxidant enzyme glutathione-S-transferase was found to be suppressed by DHA (Kim et al., 2006).

After treatment with DHA *in vitro*, the rates of proliferative inhibition of pancreatic cancer cells BxPC-3 and AsPC-1 were 76.2% and 79.5% respectively. The rates of apoptosis were increased to 55.5% and 40.0%, respectively, ($P < 0.01$ when compared with controls) (Chen et al., 2009).

DHA was shown to exhibit significant anticancer activity against the renal epithelial LLC cell line. DHA also induced apoptosis of LLC cells and influenced the expression of the vascular endothelial growth factor (VEGF) receptor KDR/flk-1. Furthermore, in both tumor xenografts, a greater degree of growth inhibition was achieved when DHA and chemotherapeutics were used in combination. The affect of DHA combined with chemotherapy on LLC tumor metastasis was significant (Zhou et al., 2010).

Artesunate (AS)

AS has been shown to inhibit the growth of Kaposi's sarcoma cells, a highly angiogenic multifocal tumor, and the degree of cell growth inhibition correlated with the induction of apoptosis. AS also inhibited the growth of normal human umbilical endothelial cells and of KS-IMM cells that were established from a Kaposi's sarcoma lesion obtained from a renal transplant patient. The inhibition of cell growth correlated with the induction of apoptosis in KS-IMM cells. Apoptosis was not observed in normal endothelial cells, which showed drastically increased cell doubling times upon AS treatment (Dell'Eva et al., 2004).

Fe(II)-glycine sulfate and transferrin enhanced the cytotoxicity (10.3-fold) of free AS, AS microencapsulated in maltosyl-ß- cyclodextrin, and ARTs towards CCRF-CEM leukemia and U373 astrocytoma cells (Efferth et al., 2004). Treatment with AS at more than 2.5 μM for 48 h inhibited the proliferation of human vein endothelial cell (HUVEC) in a concentration dependent manner using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based growth proliferation assay ($p < 0.05$). The IC_{50} value was 20.7 μM and HUVEC cells were also shown to be inhibited 88.7% by 80 μM AS (Chen et al., 2004b).

The inhibitory effect of AS on in vitro angiogenesis was tested on aortic cells cultured on a fibrin gel. AS was shown to effectively suppress the stimulating angiogenic ability of chronic myeloid leukemia cells (line K562) when the K562 cells were pretreated for 48 h with AS in a time-dependent manner (days 3-14). AS treatment was also found to decrease the VEGF level in chronic myeloma K562 cells, even at a lower concentration (2 μmol/l, $P < 0.01$). (Zhou et al., 2007).

AS at low concentration was shown to significantly decrease VEGF and Ang-1 secretion by human multiple myeloma cells (line RPMI8226, $P < 0.05$), which correlated well with the reduction of angiogenesis induced by the myeloma RPMI8226 cells. This study also showed that AS downregulated the expression of VEGF and Ang-1 in RPMI8226 cells and reduced the activation of extracellular signal regulated kinase 1 (ERK1) as well. Therefore, AS can block ERK1/2 activation, downregulate VEGF and Ang-1 expression and inhibit angiogenesis induced by human multiple myeloma RPMI8226 cells. Combined with our previous published data, results from this study indicate that AS possesses potential anti-myeloma activity (Chen et al., 2010a).

AS has been shown to decrease the secretion of VEGF and IL-8 from TNFα- or hypoxia-stimulated rheumatoid arthritis fibroblast-like synoviocyte (line RA FLS) in a dose-dependent manner. AS inhibited TNFα- or hypoxia-induced nuclear expression and translocation of HIF-1α. AS also prevented Akt phosphorylation, but there was no evidence that phosphorylation of p38 and ERK was averted. TNFα- or hypoxia-induced secretion of VEGF and IL-8 and expression of HIF-1α were hampered by treatment with the PI3 kinase inhibitor LY294002, suggesting that inhibition of PI3 kinase/Akt activation might inhibit VEGF, IL-8 secretion, and HIF-1α expression induced by TNFα or hypoxia. Therefore, AS has been shown to inhibit angiogenic factor expression in RA FLS, and this latest study provides new evidence that, as a low-cost agent, AS may have therapeutic potential for rheumatoid arthritis (He et al., 2011).

3.1.3 *In vivo* anticancer effect of ART and its derivatives

There are a small number of papers dealing with the *in vivo* anticancer activity of ARTs which may provide insight into the potential activity of ARTs *in vivo*.

Artemisinin (ART)

A study was conducted to determine the potential of ART to prevent the development of breast cancer in rats treated with 7, 12-dimethylbenz[a]anthracene (DMBA), a carcinogen known to induce multiple breast tumors. In 43% of DMBA-treated rats fed ART for 40 weeks, breast cancer tumor development was prevented, while almost all the rat fed normal food developed tumors within that time. Breast tumors of ART-fed rats were also significantly fewer and smaller in size compared with those tumors found in control animals (Lai & Singh, 2006).

Dihydroartemisinin (DHA)

DHA and ferrous sulfate have been shown to inhibit the growth of implanted fibrosarcoma tumors in rats. The growth rate of the tumors was retarded (30% less than control group) by daily oral administration of ferrous sulfate (20 mg/kg/day) followed by DHA (2–5 mg/kg/day) and no significant tumor growth inhibition was observed in the animals given either DHA or ferrous sulfate alone (Moore et al., 1995).

DHA was shown to inhibit ovarian cancer cell growth when administered alone or in combination with carboplatin, presumably through a caspase mediated apoptotic pathway. These effects were observed *in vivo* in ovarian A2780 and OVCAR-3 xenograft tumor models. DHA was shown to exhibit significant anticancer activity against ovarian cancer cells *in vivo*, with minimal toxicity to non-tumourigenic human OSE cells, indicating that DHA and ferrous sulfate may be promising therapeutic agents for ovarian cancer, either used alone or in combination with conventional chemotherapy (Chen et al, 2009).

DHA has been shown to inhibit the growth of pancreatic xenograft tumors in nude mice. The proliferation index and apoptosis index found in this study were 49.1% and 50.2% respectively in the treatment group treated with 50 mg/kg of DHA, while the proliferation index and apoptosis index of the control group was 72.1% and 9.4% respectively ($P < 0.01$). A Western blot assay conducted in the course of this study indicated that DHA up-regulated expression of the proliferation-associated protein p21(WAF1) and down-regulated expression of PCNA, increased expression of apoptosis-associated protein Bax, and decreased expression of Bcl-2 and activated caspase-9 in BxPC-3 cells. DHA was shown to exert its anti-tumor activity in pancreatic cancer both *in vitro* and *in vivo* by proliferation inhibition and apoptosis induction. The data supports the hypothesis that DHA has potential to be used as an anti-tumor drug in pancreatic cancer (Chen et al., 2009; 2010b).

Artesunate (AS)

AS has been studied in variety of tumor models as a potential antitumor drug. In one study of vascularization, a critical element of tumor metastasis, AS was shown to strongly reduce angiogenesis *in vivo* by inhibiting vascularization in Matrigel plugs injected subcutaneously into syngenic mice. This data suggests that AS represents a promising candidate drug for the treatment of the highly angiogenic Kaposi's sarcoma. As a low-cost drug, it might be of particular interest for use in areas of the world where Kaposi's sarcoma is highly prevalent. (Dell'Eva et al., 2004).

In a second study of the efficacy of AS, as an anticancer agent, tumor growth in rats given AS subcutaneously at a dose of 50 mg/kg/day and at a dose of 100 mg/kg/day for 15 days was reduced by 41%, in the 50 mg/kg treatment group and 62% in the 100 mg/kg treatment group. The density of micro-vessels which was used as a measure of angiogenic activity in the tumors of animals treated with 100 mg/kg of AS daily was at least four times lower than in the control group (Chen et al., 2004b).

In a third study, AS was also found to inhibit angiogenesis *in vivo*. The antiangiogenic activity of AS *in vivo* was evaluated in nude mice implanted with a human ovarian cancer HO-8910 cell line. The specific AS activity that inhibited angiogenesis in the ovarian tumors was determined through immunohistochemical staining for microvessel formation (CD31), VEGF and the VEGF receptor KDR/flk-1. Tumor growth was noted to be decreased and the density of the tumor microvessels was reduced following AS treatment with no apparent toxicity to the animals. ART also remarkably lowered VEGF expression in tumor cells and the expression of KDR/flk-1 in endothelial cells as well as in tumor cells (Chen et al., 2004a, 2004b).
In a fourth study, the anticancer activity of AS was correlated with the inhibition of activity in the Wnt/beta-catenin signaling pathway. *In vivo*, AS treatment resulted in a significant decrease in the rate of growth of colorectal tumor xenografts. Bioluminescent imaging also revealed that AS decreased the physiological activity of tumor xenografts and delayed spontaneous liver metastases. These antitumor effects were related to the translocation of beta-catenin to the cell membrane and the inhibition of the unrestricted activation of the Wnt/beta-catenin pathway, which was confirmed by the immunohistochemical staining of tumor tissues. These results support the use of AS for treatment of colorectal cancer and also outline a mechanism of action of AS against colorectal cancer cells (Li et al, 2007).
The antiangiogenic effect of AS was further evaluated *in vivo* in the chicken chorioallantoic membrane (CAM) neovascularization model. The results showed that stimulating angiogenic activity was decreased in response to the treatment of myeloblastic K562 cells with ART and tumor growth was inhibited when K562 cells were pretreated with ART in a dose-dependent manner (3-12 μmol/l). Furthermore, we analyzed the level of VEGF expression by Western blot and also assayed VEGF mRNA by RT-PCR in K562 cells. The experiments showed that ART could inhibit VEGF expression, and the inhibition correlated well with the level of VEGF secreted in the culture medium. These findings suggest that AS may have potential as a treatment for chronic myelogenous leukemia (CML) or as an adjunct to standard chemotherapeutic regimens (Zhou et al., 2007).
In a further study, AS inhibited the growth of ret-tumor cells and induced their apoptosis in a concentration-dependent manner (0.1-200 μmol/l). In addition, we assessed the effects of AS treatment on the immune system of treated and control animals through flow cytometric measurement quantitating different immune cell populations. No significant differences in the numbers of CD4 and CD8 T cells, T regulatory or suppressor cells, or NK cells were observed in the ret-transgenic mice and nontransgenic C57BL/6 littermates treated for 2 weeks with a daily dose of 1 mg AS. These results indicate that the cytostatic and apoptotic effects of AS are not diminished by concomitant immune suppression (Ramacher et al., 2009).
Other studies have been conducted on the successful treatment of human cancers with ART derivatives. These studies encourage further investigation of the use of ART in human cancer cases under well controlled clinical studies (Berger et al., 2005; Singh & Panwar, 2006).

3.2 Mechanistic perspectives on the antiangiogenic activities of ARTs

Angiogenesis and vasculogenesis refer to the growth of blood vessels. Angiogenesis is the growth most often associated with repair of damaged vessels or the growth of smaller blood vessels, while vasculogenesis is the process by which the primary blood system is being created or changed. Vasculogenesis occurs during the very early developmental stages of an organism when the blood vessel pathways are created. Angiogenesis, while a similar process, does not depend on the same set of genes as vasculogenesis, and this process is

activated instead in the presence of an injury to a blood vessel. In the last three decades, considerable research has been reported that supports the hypothesis that tumor growth and metastasis require angiogenesis. Angiogenesis, the proliferation and migration of endothelial cells resulting in the formation of new blood vessels, is an important process for the progression of tumors (Figure 2). ARTs have been shown in a number of published papers to have antiangiogenic effects.

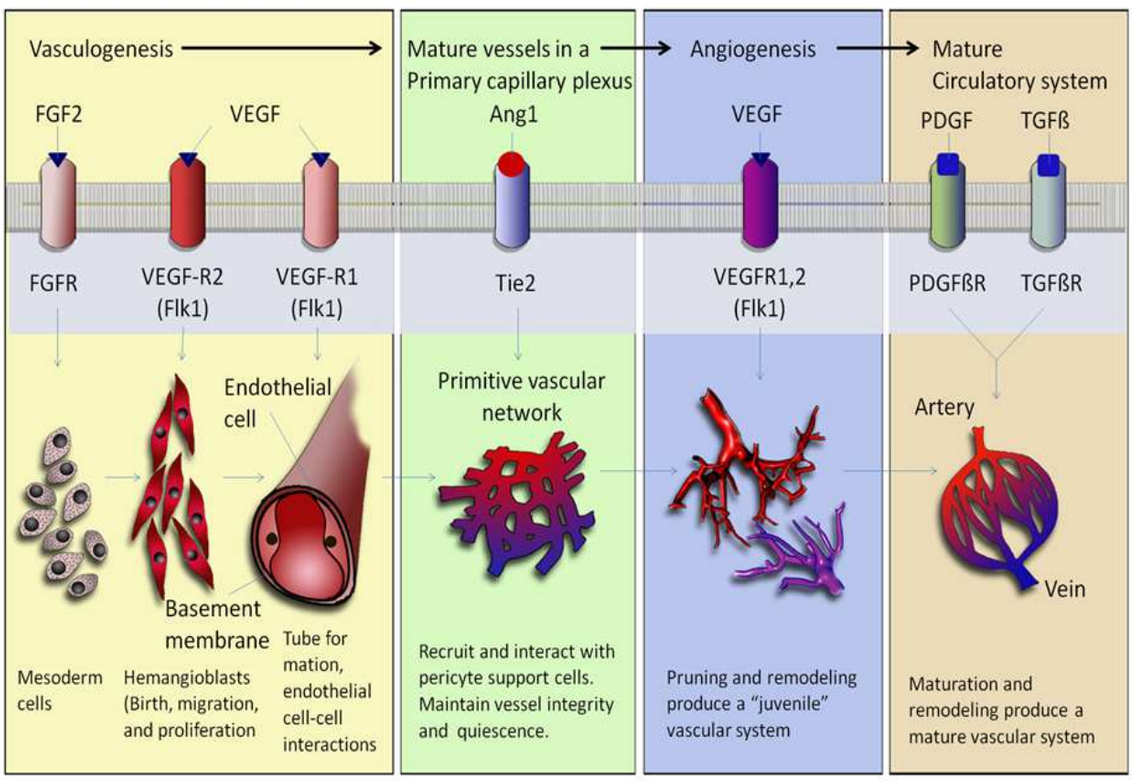

Fig. 2. The modes of vasculogenesis and angiogenesis. Vasculogenesis occurs during the very early developmental stages of an organism when the blood vessel pathways are created. Angiogenesis, while a similar process, does not depend on the same set of genes as vasculogenesis, and this process is activated instead in the presence of an injury to a blood vessel. Angiogenesis finishes the circulatory connections begun by vasculogenesis and builds arteries and veins from the capillaries. In this diagram, the major paracrine factors involved in each step are shown boxed, and their receptors (on the vessel-forming cells) are shown beneath them. (Modified from Hanahan, 1997)

3.2.1 Inhibition of vasculogenesis and angiogenesis leads to embryotoxicity

Given the potent effects of ART and its derivatives on inhibition of angiogenesis, it is perhaps not surprising that these compounds have been reported to be embryotoxic in rodents. In several reports, DHA was shown to cause significant embryotoxicity accompanied by developmental defects in the neural tube, branchial arches, somites and caudal region in rat embryos *in vitro*. This finding has great significance for potential *in vivo* effects as DHA has been shown to cross embryonic membranes, and the embryonic yolk sac has also been shown to be highly susceptible to ART compounds (Longo et al., 2006a; 2006b; White et al., 2008).

Previous data has shown the rapid onset of action of AS and DHA on primitive RBCs as soon as they enter embryonic circulation. There is no clear explanation as to why primitive RBCs are susceptible to DHA (Longo et al., 2006a, 2006b). Like intraerythrocytic malaria parasites, primitive RBCs have high concentrations of iron and heme, which have been proposed as either activators or targets of ART compounds (Olliaro et al., 2001; Parapini et al., 2004). The depletion of embryonic erythroid cells by ART compounds likely occurs as a consequence of the inhibition of vasculogenesis. This is a plausible hypothesis because the processes of vasculogenesis and hematopoiesis are actually strongly related. Formation of a blood vessel is accompanied by the simultaneous in situ production of blood cells within that vessel (Baron 2001; Baumann & Dragon, 2005; Sequeira Lopez et al., 2003). One additional study that supports this hypothesis involved the effects of AS on embryo development. In this study, oral and injectable AS were shown to induce marked embryo lethality accompanied by a low incidence of teratogenic effects, including cardiovascular defects (ventricular septal and great vessels defects), which significantly affected novel vessel formation (Clark et al., 2004, White et al., 2008; Ratajska et al., 2006a, 2006b).

3.2.2 Anti-VEGF of ARTs plays a key role during normal and pathological angiogenesis

Angiogenesis is promoted by numerous factors including cytokines such as VEGF, bFGF, PDGF and others. It is negatively regulated by angiostatin, endostatin, thrombospondin, TIMPs and other factors. The factors that are produced in tumor cells as well as in surrounding stromal cells act in a balance to promote either proangiogenic or antiangiogenic processes. Among the cytokines for regulating angiogenesis, VEGF and angiopoietin-1 (Ang-1) have specific modulating effects on the growth of vascular endothelial cells, and they play a key role in the process of angiogenesis (Thurston, 2002).

VEGF is a homodimeric 34-42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. Two receptor tyrosine kinases have been described as putative VEGF receptors, Flt-1 and KDR. Flt-1 (fms-like tyrosine kinase), and KDR (kinase-insert-domain-containing receptor) proteins have been shown to bind VEGF with high affinity.

In vitro, VEGF is a potent endothelial cell mitogen. In cultured endothelial cells, VEGF has been shown to activate phospholipase C and induce rapid increases of free cytosolic Ca^{2+}. VEGF has also been shown to stimulate the release of von Willebrand factor from endothelial cells and induce expression of tissue factor activity in endothelial cells as well as in monocytes. VEGF has also been shown to be involved in the chemotaxis of monocytes and osteoblasts. *In vivo*, VEGF can induce angiogenesis as well as increase microvascular permeability. As a vascular permeability factor, VEGF acts directly on the endothelium and does not degranulate mast cells. It promotes extravasation of plasma fibrinogen, leading to fibrin deposition which alters the tumor extracellular matrix. The modified extracellular matrix subsequently promotes the migration of macrophages, fibroblasts and endothelial cells. Based on its *in vitro* and *in vivo* properties, VEGF is believed to play important roles in inflammation and also in normal and pathological aspects of angiogenesis, a process that is associated with wound healing, embryonic development, growth, and metastasis of solid tumors. Elevated levels of VEGF have been reported in synovial fluids of rheumatoid arthritis patients and in sera from cancer patients.

Over the last three decades, a growing body of evidence has developed on the role of angiogenesis in tumor growth and metastases of tumors (Firestone & Sundar, 2009).

Angiogenesis can be divided into a series of temporally regulated responses, including induction of proteases, migration of endothelial cells, cell proliferation and differentiation. This is a highly complex process, in which a number of cytokines and growth factors released by endothelial cells, tumor cells and matrix cells are involved. The expression of VEGF has been suggested to be related to some fundamental features of solid tumors, such as the growth rate, the density of tumor microvessels, and the development of tumor metastases

3.2.3 Antiangiogenic mechanisms of ARTs

Pathological angiogenesis, the formation of new blood vessels from pre-existing ones in tumors, is essential for supplying tumors with oxygen and nutrients and critical for the spread of metastatic cells throughout the body. Inhibitors of angiogenesis that block angiogenic signals have been developed, and antiangiogenic therapy strategies have been shown to be valuable adjuncts to cytostatic and cytotoxic chemotherapy (Efferth 2005).

3.2.3.1 Effect of ARTs on angiogenesis-related genes

Establishment of a network of vasculature in a tumor is a critical event for tumor growth and survival. This process is accomplished by a complex sequence of temporal events involving vasculogenic secretions from tumor cells, restructuring of the extracellular matrix using matrix metalloproteinases and formation of new vasculature. Because angiogenesis involves tissue restructuring, genes that regulate angiogenesis, such as chemokine receptors, can also affect tumor metastasis (Wu et al., 2009). In a study conducted with an NCI panel of 60 tumor cell lines, treatment of these cells with ART and related compounds resulted in altered expression of genes implicated in angiogenesis suggesting antiangiogenic activity (Anfosso et al., 2006).

In this study, microarray analysis of mRNA expression of 30 out of 89 angiogenesis-related genes correlated significantly with the cellular response to several ARTs. Among this panel of genes were many fundamental angiogenic regulators such as vascular endothelial growth factor C (*VEGFC*), fibroblast growth factor-2 (*FGF2*), matrix metalloproteinase 9 (*MMP9*), thrombospondin-1 (*THBS1*), hypoxia-inducing factor-α (*HIF1a*), angiogenin (*ANG*) and others. By means of hierarchical cluster analysis, expression profiles were identified that demonstrated significant cellular responses to AS, arteether, artemether, and dihydroartemisinylester stereoisomer 1. A borderline significance was observed after treatment with dihydroartemisinylester stereoisomer 2 and ART (Efferth 2005). The sensitivity and resistance of these tumor cells correlated with the mRNA expression of angiogenesis-related genes. This suggests that that the anti-tumor effects of ARTs are likely due to their role in inhibiting tumor angiogenesis (Anfosso et al., 2006).

3.2.3.2 ART treatment leads to decreases in expression levels of HIF-1a

Tumor hypoxia activates the transcription factor hypoxia inducible factor-1α (HIF-1α). This adaptation increases tumor angiogenesis to support the survival of poorly nourished cancer cells. Hypoxic tumors are resistant to radiation and many anti-cancer agents. HIF-1α is activated during angiostatic therapy, and HIF-1α has also been shown to up-regulate the expression of transferrin receptors. Since ART is selectively toxic to iron-loaded cells, radio and drug-resistant tumors might be selectively susceptible to attack by a treatment strategy consisting of iron-loading and ART treatment (Efferth 2005).

These findings are consistent with previous findings (Wartenberg et al., 2003) that noted ART-dependent decreases in expression levels of HIF-1a. HIF-1a is known to be a

transcriptional activator of VEGF, and it plays a crucial role in neo-vasculogenesis in hypoxic tissues. ART treatment of leukemic and glioma cells *in vitro* at a concentration of 12 mM was shown in another study to inhibit angiogenesis. This ART driven angiogenesis inhibition was shown to involve suppression of VEGF and HIF-1a expression at the transcriptional level. (Huang et al., 2008; Zhou et al., 2007). Loss of HIF-1a and VEGF expression after ART treatment appears to be dependent on production of ROS because co-treatment with free-radical scavengers such as vitamin E and mannitol reversed the effects of ART (Wartenberg et al., 2003).

3.2.3.3 Anti-VEGF activities of ARTs

1. ART decreases expression of VEGF and alters VEGF receptor binding

ART and DHA have been shown to significantly inhibit angiogenesis in a dose-dependent manner as demonstrated by measurement of the proliferation, migration and tube formation of human umbilical vein endothelial (HUVE) cells (Chen et al., 2003). DHA was shown to markedly reduce VEGF binding to its receptors on the surface of HUVE cells and reduced the expression levels of two major VEGF receptors, Flt-1 and KDR/flk-1, on HUVE cells. Chicken chorioallantoic membrane (CAM) neovascularization was significantly inhibited by DHA (Chen et al., 2004a). The inhibitory effect of ART on HUVE cell proliferation was stronger than the effect of ART on HO-8910 cancer cells, NIH-3T3 fibroblast cells or human endometrial cells (Chen et al., 2004b). ART derivatives also inhibited HUVE cell tube formation and exhibited antiangiogenic effects (Oh et al., 2004).

In addition to affecting expression of the VEGFs, ART and its derivatives have been shown to target the VEGF receptors. In an ovarian cancer xenograft model, treatment with 50 mM AS resulted in the inhibition of microvessel formation, and immunohistochemical staining revealed that AS treated xenografts displayed significantly reduced levels of CD31 (a neovasculogenesis marker) and the VEGF receptor KDR. AS also inhibited VEGF-induced migration and differentiation of cultured human umbilical vascular endothelial cells (Chen et al., 2009).

2. Torilin, a related sesquiterpene, inhibits blood vessel formation

It is interesting to note that torilin, another sesquiterpene (derived from the fruits of *Torilis japonica)*, has also been shown to be a potent antiangiogenic factor which also inhibits blood vessel formation by disrupting VEGFA expression. A similar finding was also shown by using DHA (Kim et al., 2000; 2006). Hence, the ability of ART to inhibit angiogenesis may be due to its chemical nature as a sesquiterpene. Another compelling finding is that other phytosesquiterpene lactones, such as costunolide from *Saussurea lappa*, can inhibit KDR signaling (Jeong et al., 2002). Comparisons with other sesquiterpenes may shed more light on the unique features of the anticancer actions of ART, and potentially lead to better angiostatic drug design

3. ARTs down-regulate expressions of HIF-1a and VEGF

Wartenberg et al. (2003) investigated the anti-angiogenic effects of ART on mouse embryonic stem cell-derived embryoid bodies, which are a model system for early post implantation embryos which differentiate well into capillaries. ART dose-dependently inhibited angiogenesis in embryoid bodies and raised the level of intracellular reactive oxygen species. Furthermore, ART treatment was shown over the time course of embryoid body differentiation to impair organization of the extracellular matrix laminin component and altered expression patterns of matrix metalloproteinases 1, 2, and 9. Analysis of mRNA expression in embryoid bodies showed that ART treatment resulted in the down-regulation-of HIF-1α and VEGF, both of which control endothelial cell growth.

4. ART inhibits chicken chorioallantoic membrane (CAM) angiogenesis

By utilizing the chicken CAM culture technique, it is possible to detect the microangium-like structures formed by *in vitro* cultivated arterial rings. Through this method, AS has been shown to also have antiangiogenic effects. AS treatment significantly inhibited chicken CAM angiogenesis, proliferation and differentiation of human microvascular dermal endothelial cells in a dose-dependent manner and reduced Flt-1 and KDR/flk-1 expression (Huan-Huan et al., 2004). AS was shown to strongly reduce angiogenesis *in vivo* as shown by changes in vascularization of Matrigel plugs injected subcutaneously into syngenic mice (Dell'Eva et al., 2004). AS has also been shown to retard the growth of human ovarian cancer HO-8910 xenografts in nude mice. In this study, microvessel density was reduced following AS treatment with no apparent animal toxicity. In addition, AS treatment also markedly lowered VEGF expression in tumor cells and KDR/flk-1 expression in endothelial cells as well as tumor cells (Chen et al., 2004a).

Through a human umbilical vein endothelial cell (HUVEC) injury migration experiment, AS has been shown to inhibit the multiplication, migration and cannulation of endothelial cells, and AS was shown to effectively suppress the genesis and growth of tumor vessels. The tumor angiogenesis and growth inhibitory effect of ART was also shown by Chen, et al. (2004a), and results of their study showed that ART could inhibit the production of VEGF and its receptor, KDR/flk-1, in tumor cells, and AS was also shown to induce cellular apoptosis in oophoroma, a rare ovarian tumor. In experimental studies using CAM and aortic ring non-serum cultures, the secretion of VEGF was monitored using ELISA testing. AS treatment of CAM cultures at a concentration as low as 2 mmol/L was shown to reduce the level of VEGF, effectively inhibiting angiogenesis in co-cultured chronic myelocytic leukemia K562 cells (Lee et al., 2006; Zhou et al., 2007). In summary, these experimental studies have provided a wealth of evidence to support the hypothesis that ART treatment can effectively inhibit leukemia cell proliferation and inhibit angiogenesis of solid tumor cells. The mechanisms of this anticancer activity include direct inhibition of tumor cell multiplication, induction of apoptosis, the inhibition of angiogenesis through suppression of VEGF secretion, and inhibition of VEGF receptor expression.

3.3 Anticancer case reports and clinical trials of ARTs in humans

Clinical evidence has accumulated showing that artemisinin-derived drugs have promise for treatment of laryngeal carcinomas, uveal melanomas and pituitary macroadenomas. ART compounds are also in phase I-II trials against breast, colorectal and non-small cell lung cancers (Table 3).

Artemisinins	Cancer targets	Clinical studies	Protocols & References
Artemisinin	Colorectal cancer	Clinical trial, Phase I	ISRCTN05203252, 2011 UK
Artesunate	Non-small cell lung cancer	Clinical trial Phase I-II	Zhang et al., 2008 CHINA
	Metastatic uveal melanoma	Case report	Berger et al., 2005 GERMANY
	Laryngeal carcinoma	Case report	Singh & Verma, 2002 INDIA
	Metastatic breast cancer	Clinical Trial, Phase I-II	NCT00764036 2008 GERMANY
Artemether	Pituitary macroadenoma	Case report	Singh& Panwar 2006 INDIA

All clinical trials listed here are completed.

Table 3. Anticancer effects of artemisinin (ART), artesunate (AS), and artemether (AM) in case reports of treatments and clinical trials (Ghantous et al., 2010)

3.3.1 Case reports

1. Metastatic uveal melanomas treated with AS

Berger et al. reported on the first long-term treatment of two cancer patients with AS in combination with standard chemotherapy. These patients with metastatic uveal melanoma were treated on a compassionate-use basis, after standard chemotherapy alone was ineffective in stopping tumor growth. The therapy regimen was well tolerated with no additional side effects other than those caused by standard chemotherapy alone. One patient experienced a temporary response after the addition of AS to Fotemustine while the disease was progressing under therapy with Fotemustine alone. The second patient first experienced a stabilization of the disease after the addition of AS to Dacarbazine, followed by objective regressions of splenic and lung metastases. This patient is still alive 47 months after first diagnosis with stage IV uveal melanoma, a diagnosis with a median survival of 2-5 months.

Despite the small number of treated patients, AS may be a promising adjuvant drug for the treatment of melanoma and possibly other tumors in combination with standard chemotherapy. AS is well tolerated, and the lack of serious side effects will facilitate prospective randomized trials in the near future. From *in vitro* studies already conducted (Efferth et al., 2004), it is further conceivable that loading tumor cells with bivalent iron, by simply providing Fe^{2+} in tablet form, might increase the susceptibility of cancer cells to AS treatment. It is tempting to speculate that, in the case of the second patient previously discussed, the addition of Fe^{2+} had an actual clinical impact and resulted in an improved response to therapy (Berger et al., 2005).

2. Laryngeal carcinoma treated with AS

AS injections and tablets were used in one study to treat a laryngeal squamous cell carcinoma patient over a period of nine months. The tumor was significantly reduced in size by 70% after two months of treatment. Overall, the AS treatment of the patient was beneficial in prolonging and improving quality of life. Without treatment, laryngeal cancer patients die within an average of 12 months. The patient lived for nearly one year and eight months until his death due to pneumonia.

The observations that the patient regained his voice, appetite, and weight after a short term treatment with AS, and the fact that the tumor was significantly reduced in size without any apparent adverse side effects suggests that AS treatment could be an effective and economical alternative treatment for cancer, especially in cases of late cancer detection where available treatments are limited. Since this case report was published, several patients with different types of cancers have begun treatment with artemisinin and its analogs with promising results. AS therapy has potential to prevent and treat a wide range of cancers given its efficacy, low cost, and due to the common mechanisms of action demonstrated against various cancer cells (Singh & Verma, 2002).

3. Pituitary macroadenoma treated with artemether

Artemether, an ART analogue, was used to treat a 75-year old male patient with pituitary macroadenoma. This patient presented with vision, hearing, and locomotion-related problems as a consequence of his disease. Artemether was administered orally to the patient over a period of 12 months. Although the tumor remained consistent in size, CT scans showed a reduction in tumor density, and clinically, the related symptoms and signs improved significantly as therapy progressed. Overall, the artemether treatment was beneficial in improving the patient's quality of life. Artemether and other artemisinin analogs appear to have promise for treatment of this type of cancer (Singh and Panwar, 2006).

3.3.2 Clinical trials of ARTs as anticancer agents

1. Phase I study of oral AS to treat colorectal cancer (Completed)

The primary objective of this study was to determine the effects of oral AS in inducing apoptosis in patients awaiting surgical treatment of colorectal adenocarcinoma. The secondary objective of this study was to establish the tolerability of oral AS for the treatment of colorectal cancer. Subjects were randomized to receive either 200 mg AS or placebo orally once daily for 14 days while awaiting surgery for definitive surgical treatment of colorectal adenocarcinoma. A significant difference in the proportion of colorectal adenocarcinoma cells exhibiting apoptosis was noted between the two treatment groups (placebo and AS), assessed at the time of surgery after two weeks of drug treatment. No result was publicly issued (Protocol Number: ISRCTN05203252).

2. Phase II study of AS treatment as an adjunct to treat non-small cell lung cancer (Completed)

This study was designed to compare the efficacy and toxicity of AS treatment combined with NP (a chemotherapy regimen of vinorelbine and cisplatin) and NP alone in the treatment of advanced non-small cell lung cancer (NSCLC). One hundred and twenty cases of advanced NSCLC were randomly divided into an NP chemotherapy group and a combined AS with NP therapy group. Patients in the control group were treated with the NP regimen of vinorelbine and cisplatin. Patients in the trial group were treated with the NP regimen supplemented with intravenous AS injections (120 mg, once-a-day intravenous injection, from the 1st day to 8th day, for 8 days). At least two 21-day-cycles of treatment were performed. There were no significant differences in the short-term survival rates, mean survival times and the 1-year survival rates between the trial group and the control group, which were 44 weeks and 45 weeks, respectively. The disease controlled rate of the trial group (88.2%) was significantly higher than that of the control group (72.7%) ($P < 0.05$), and the trial group's time to disease progression (24 weeks) was significantly longer than that of the control group (20 weeks). No significant difference was found in toxicity between the two treatment groups. Therefore. AS combined with NP can increase the disease controlled rate and prolong the time to progression of patients with advanced NSCLC without significant side effects (Zhang et al., 2008).

3. Phase I study with metastatic breast cancer (Completed)

The purpose of this study was to evaluate the tolerability of an adjunctive therapy with AS for a period of 4 weeks in patients over the age of 18 years with advanced metastatic breast cancer, which was defined as a histologically or cytologically confirmed. Women of childbearing potential were tested to rule out pregnancy prior to their treatment. Relevant neurological symptoms, adverse events, and the relation between adverse events and the use of AS, as an adjunct, saliva cortisol profile, overall response rate, clinical benefit, and assessment of patients' expectations will be monitored as study endpoints. No result of this study has yet been publicly issued (Protocol Number: NCT00764036).

4. Therapeutic implications of ARTs due to alternative vascularization mechanisms

Until recently, normal and abnormal processes of vascularization (vasculogenesis and angiogenesis) were considered to be based on a limited number of known mechanisms. Recent advances have been made in identifying a number of novel alternate processes involved in vasculogenesis and angiogenesis. If these new findings of alternate mechanisms are confirmed, cancer therapy strategies may also be affected

4.1 The role of the visceral yolk sac endoderm in primitive erythropoiesis

The role of the visceral yolk sac endoderm in the control of primitive erythropoiesis and vasculogenesis remains a subject of debate. During mouse embryogenesis, the first hematopoietic and endothelial cells form in blood islands located between layers of visceral endoderm and mesoderm in the yolk sac. One study assessed the consequences of the absence of a visceral endoderm layer on blood cell and vessel formation using embryoid bodies derived from mouse embryonic stem (ES) cells deficient in GATA-4, a transcription factor expressed in the yolk sac endoderm.

When differentiated *in vitro*, these mutant embryoid bodies did not develop an external visceral endoderm layer. GATA-4 deficient embryoid bodies (GATA 4-1 ES cells), grown either in suspension culture or attached to a substratum, were shown to be defective in primitive hematopoiesis and vasculogenesis as evidenced by a lack of recognizable blood islands, vascular channels, and a reduction in the expression of primitive erythrocyte markers. Expression of the endothelial cell transcripts for Flk-1, Fit-1, and platelet-endothelial cell adhesion molecule (PECAM) was not affected in the mutant embryoid bodies. Gata4-1-ES cells retained the capacity to differentiate into primitive erythroblasts and endothelial cells when cultured in methylcellulose or Matrigel. Analysis of chimeric mice, generated by injecting Gata4-l-ES cells into 8-cell stage embryos of ROSA26 transgenic animals, showed that Gata4-l-ES cells can form blood islands and vessels when juxtaposed to visceral endoderm *in vivo*. The authors of this study concluded that the visceral endoderm is not essential for the differentiation of primitive erythrocytes or endothelial cells, but this cell layer plays an important role in the formation and organization of yolk sac blood islands and vessels (Bielinska et al., 1996).

4.2 Origin of the first definitive erythropoiesis

Although primitive and definitive blood cells arise at separate locations in advanced stage embryos, some experimental evidence suggests that cell migration may occur between the blood-forming compartments. Thus, the origin of stem cells for multi-lineage hematopoiesis has been a controversial issue in the field. Studies from amniotes have linked the first stem cell activity to the aorta-gonad-mesonephros (AGM) region, whereas others suggest that the yolk sac is the true source of hematopoietic stem cells.

The oldest hypothesis is based on the thought that all hematopoietic stem cells originate from the yolk sac. The most relevant supporting evidence for this hypothesis is based on the observation that host chick bodies grafted on to a donor yolk sac contain cells from the yolk sac in their hematopoietic organs. Additional work conducted more recently has focused on the use of molecular tools to characterize the underlying events that initiate erythropoiesis in vertebrate embryos. Several key genes have been identified that are necessary for primitive and subsequent definitive erythropoiesis, which differs in several aspects from primitive erythropoiesis (Baumann and Dragon, 2005). This data is informative, but it is also clear that more physiological data are needed to understand in detail the function of embryonic hemoglobin and primitive RBCs. These reports offer an interesting perspective for future study to answer two questions: 1) what are the molecular determinants for the initiation of erythropoiesis? and 2) what are the physiological functions of early erythroid cells?

4.3 Erythropoiesis in the embryonic heart

Another possible explanation of early erythropoiesis is in situ differentiation from progenitor cells that migrate to the embryonic heart at the onset of vascularization. The embryonic heart has been postulated to be a hematopoietic organ in previous reports supporting the hypothesis

that hematopoiesis within the embryo is strictly limited to the areas of vasculogenesis. However, a separate study on this issue showed the absence of hematopoietic stem cells in the embryonic heart by both transmission electron microscopy (TEM) and also through immunohistochemical staining with antibodies to hematopoietic stem cell (HSC) antigens. Investigators performing this study also could not find any evidence for the presence of blood islands exhibiting a pattern of cellular assembly similar to a yolk sac blood island. This study suggests that the embryonic heart supplies only new erythroblasts owing to their proliferative capacity within the primitive vascular vesicles at the time before coronary vessels are connected to the aorta (Ratajska et al., 2006b). It is doubtful that the embryonic heart possesses a hematopoietic activity since this activity is always associated with production of a wealth of descendent cells. Since formation of blood island-like structures occurs throughout prenatal life (Rongish et al., 1994), it is possible that red blood cells (nucleated or enucleated) enter the embryonic heart also at later stages of development (Ratajska et al., 2006b).

4.4 Glioblastoma vascularization formed by vasculogenic mimicry

EL Hallani et al. (2010) described a new mechanism of alternative glioblastoma vascularization and opened a new perspective for an antivascular treatment strategy. Glioblastomas are the most frequent and malignant primary brain tumors in adults and have a poor prognosis despite surgery and conventional radio-chemotherapy. Histologically, glioblastomas are highly angiogenic and are characterized by microvascular proliferations (Louis et al., 2007). Antivascular endothelial growth factor therapy has demonstrated significant efficacy in treatment of glioblastomas with nearly 50% of treated patients responding to therapy, but it is still possible for these tumors to acquire antiangiogenic resistance (Kreisl et al., 2009). It is known that alternative vascularization mechanisms may occur in brain tumors, such as co-opting of existing vessels, angioblast vasculogenesis, intussusceptive microvascular growth and vasculogenic mimicry. The term vasculogenic mimicry describes the formation of fluid-conducting channels by highly invasive and genetically dysregulated tumor cells. Two distinctive types of vasculogenic mimicry have been reported in tumors, vascular mimicry of the patterned matrix type and vascular mimicry of the tubular type.

Vasculogenic mimicry of the patterned matrix type results in the ability of tumor cells to express endothelium-associated genes that are also involved in embryonic vasculogenesis. Such plastic properties could be associated with cancer stem cells, a subpopulation of undifferentiated tumor cells that present with a marked capacity for proliferation, self-renewal, multiple lineage differentiation and tumor initiation. This finding provides a better understanding of the process of tumor vascularization involving cancer stem-cell plasticity, and it has important implications in determining a proper treatment strategy. The evaluation of the overall contribution of such tumor cell-formed vessels to glioblastoma blood flow should be based on the sensitivity of gliomas to current antiangiogenic therapies using quantitative methods with appropriate sampling. Also, understanding the influence of the microenvironment in determining the vascular fate of glioblastoma cells may provide new perspectives on tumor cell plasticity that could be exploited for novel strategies in cancer differentiation therapy (El Hallani et al., 2010).

4.5 Vascularization mechanism in cancer pathology

Before discussing the different ways a tumor is vascularized, we should emphasize that these mechanisms are not mutually exclusive; in fact, in most cases they are interlinked, being involved concurrently in physiological as well as in pathological angiogenesis. Although the molecular regulation of endothelial sprouting has been extensively studied

and reviewed in the literature, the morphogenic and molecular events associated with alternative cancer vascularization mechanisms are less understood. Cancer cells are not generally controlled by normal regulatory mechanisms, but tumor growth is highly dependent on the supply of oxygen, nutrients, and host-derived regulators. It is now established that tumor vasculature is not necessarily derived from endothelial cell sprouting. Cancer tissue can acquire vasculature by a variety of mechanisms to include co-opting pre-existing vessels, intussusceptive microvascular growth, postnatal vasculogenesis, glomeruloid angiogenesis, or vasculogenic mimicry. The best-known molecular pathway driving tumor vascularization is the hypoxia-adaptation mechanism. Other pathways involving a broad and diverse spectrum of genetic aberrations, however, are associated with the development of the "angiogenic phenotype." Based on this knowledge, novel forms of antivascular modalities have been developed in the past decade.

When applying these targeted therapies, the stage of tumor progression, the type of vascularization of the given cancer tissue, and the molecular machinery behind the vascularization process all need to be considered. A further challenge is finding the most appropriate combinations of antivascular therapies and standard radio- and chemotherapies. The most promising therapeutic plan of action will involve the integration of our recent knowledge in this field into a rational strategy to for developing effective clinical modalities using antivascular therapy for cancer (Döme et al., 2007).

4.6 Genetic effects of ARTs contribute to sensitivity of cancer cells to chemotherapy

Endothelial cells involved in vasculogenesis and angiogenesis are key targets in cancer therapy. Recent evidence suggests that tumor cells can express some genes typically expressed by endothelial cells and form extracellular matrix–rich tubular networks, a phenomenon known as vasculogenic mimicry. Schaft et al. (2004) examined the effects of three angiogenesis inhibitors on vasculogenic mimicry in human melanoma MUM-2B and C8161 cells and compared them with their effects in human endothelial HMEC-1 and HUVEC cells. Their data reveals biologically significant differences in the responses of endothelial cells and aggressive melanoma cells that are engaged in vasculogenic mimicry to select angiogenesis inhibitors. Because vasculogenic mimicry has been reported in several other tumor models, including breast, prostatic, ovarian, and lung carcinoma (Hendrix et al. 2003), these findings may contribute to the development of new antivascular therapeutic agents that target both angiogenesis and tumor cell vasculogenic mimicry.

Similar analyses have identified angiogenesis-related genes that are differentially expressed in AS-sensitive and resistant cell lines (Anfosso et al., 2006). The sensitivity of cells to AS therapy was shown to correlate with cell viability, growth and an angiogenic phenotype. Resistance to AS treatment to inhibit growth would also thus extend to an antiangiogenic response of an AS-resistant tumor cell in its microenvironment. It is therefore probable that these genes associated with AS resistance also determine the antiangiogenic response of the cell lines when treated with AS. Anfosso et al. (2006) have shown that a panel of genes that correlate with the cellular response to AS contains many fundamental angiogenic regulators, such as the vascular endothelial growth factors, which stimulate proliferation and migration of endothelial cells, a fundamental step in vessel formation. Three human genes encode for vascular endothelial growth factors (VEGFA, VEGFB, and VEGFC). The investigators decided to include in the cluster analysis only those genes whose mRNA expression correlated with GI_{50} values (the concentration needed to inhibit the growth of treated cells to half that of untreated cells) of at least four ARTs. After this truncation of the gene panel, only VEGF-C remained as an angiogenic regulator among the 30 genes in the cluster analysis panel.

Through knockout studies in mice, a number of genes participating in yolk sac hematopoiesis and vasculogenesis have been identified. Some of these gene disruptions affect only hematopoiesis, while other gene disruptions were shown to disturb vascular development, and still others were shown to affect both processes. Additional factors influencing yolk sac vasculogenesis and hematopoiesis are likely to emerge in the coming years. Gata4-1 embryoid bodies will provide a useful visceral endoderm free system in which to study the effects of growth or differentiation factors normally produced by the visceral endoderm, including substances that affect primitive hematopoiesis and vasculogenesis (Bielinska et al., 1996).

The antiangiogenic activities of both ART and AS have been investigated by a number of researchers. ART has been shown to downregulate vascular endothelial growth factor (VEGF) expression, an effect that was reversed upon co-treatment with the free radical scavengers mannitol and vitamin E. This indicates that ART may act in an antiangiogenic manner via generation of reactive oxygen species. AS and DHA have been shown to reduce the expression of the two major VEGF receptors, Flt-1 and KDR/flk-1, as determined by immune histochemistry in the chicken chorioallantoic membrane neovascularization model, in HUVE cells , and in nude mice injected with the human ovarian cancer line HO-8910, respectively. The results of these authors and others suggest that the antiangiogenic effect induced by ARTs might occur by induction of cellular apoptosis and inhibition of expression of VEGF receptors (Chen et al., 2004a; Oh et al., 2004; Wartenberg et al., 2003).

5. Further development of ARTs as antiangiogenic cancer agents

Cancer angiogenesis has been confirmed by measurement of high proliferation indices for endothelial cells, not only in rapidly growing animal tumors, but also in human tumors. The rationale for developing antiangiogenic strategies for cancer therapy was based on the fact that physiological angiogenesis only occurs in a limited number of situations, such as wound healing and the menstrual cycle. This suggests there is an opportunity for developing highly tumor-specific antiangiogenic applications which utilize drugs such as the ARTs which have demonstrated antiangiogenic efficacy with little toxicity.

5.1 Prevention and therapy strategies of ARTs as anticancer agents

The tumor vasculature is an attractive target for cancer therapy because of its accessibility to blood-borne anticancer agents and the reliance of most tumor cells on an intact vascular supply for their survival. Therapeutic targeting of the tumor vasculature can be divided into two approaches, an antiangiogenic approach and an antivascular approach. Antiangiogenic approaches are focused on disrupting the processes involved in the outgrowth of new blood vessels from pre-existing ones, while antivascular approaches are targeted to affect the established tumor vasculature. Individual agents may possess both antiangiogenic and antivascular properties. However, a practical distinction between the two approaches can be made based on the dosing strategies employed. In order to prevent angiogenesis, a chronic dosing schedule is appropriate, whereas single dose or split dose treatments are more effective for antivascular activity, which is targeted to rapidly shut-down blood flow in established tumor blood vessels.

Today the angiosuppressive strategy is the most developed, containing a variety of agents. The first class of angiosuppressive agents developed targets the primary angiogenic cytokine in cancer, VEGF, by monoclonal antibodies, which act to trap VEGF (so called VGF trap agents) or antisense antibodies directed at VEGF (VEGF-antisense agents). The second approach is to

target VEGF receptors through the use of monoclonal antibodies. Interestingly, anti-receptor antibody therapy is in the early phase of development, and most of the available agents are small molecular VEGF receptor signal transduction inhibitors. Since VEGF is not the only pro-angiogenic cytokine produced by cancers, it will likely be necessary to develop other anti-angiogenic agents , but these targets and the appropriate agent to block either a novel cytokine or its receptor are unknown today (Tímár & Döme, 2008).

In contrast to the antiangiogenesis approach, antivascular strategies aim to cause a rapid and extensive shut-down of the established tumor vasculature, leading to secondary tumor cell death. Cell death following blood flow shut-down, induced by clamping or ligation of the tumor-supplying blood vessels, is characterized by an early and extensive tumor cell necrosis (Tozer, 2003). Therefore, this pattern of cell death following treatment is indicative of vascular-mediated cytotoxicity. There is potential for specific targeting of the tumor vasculature based on selective expression of proteins on tumor endothelial cells. Recent development of techniques for the isolation of tumor-derived endothelial cells and gene expression has led to the identification of a number of gene transcripts which are specifically elevated in tumor-associated endothelium. Antivascular approaches under investigation include integrin-binding peptides conjugated to anticancer drugs, antibodies targeted to endothelial-specific proteins, and gene therapy approaches (Tozer, 2003).

5.2 Combination strategies to enhance efficacy of ARTs as anticancer agents

There is growing evidence supporting the use of ART and its derivatives in cancer therapy. ARTs are a class of compounds that are first-line treatment options for malaria. They also have potent antiproliferative, antimetastatic and antiangiogenic activity, which makes them potential anticancer drugs (Liu et al., 2011). Scientists investigating the cancer-fighting properties of AS have found early evidence that combining it with an existing cancer drug has the potential to make each drug more effective than when used alone. There is currently limited published data exploring the value of ART as a combination partner in treatment regimens. These studies have used simple approaches to studying drug–drug interactions, and as a consequence, their conclusions are still open to debate. The idea of combining drugs in therapeutic regimens is to achieve an overall effect that is greater than the sum of the individual effects of each agent (Liu, 2008).

Drug combinations that involve ART have been reported *in vitro*, which show value in this approach, both as a sensitizing agent to chemotherapy in solid tumors (Hou et al., 2008; Sieber et al., 2009), and as a synergistic partner with doxorubicin in leukemia (Efferth et al., 2007). Incubation of cancer cells with DHA alone was found to be less effective than in combination with holotransferrin, indicating that intracellular iron plays a role in the cytotoxic effects of DHA (Lai & Singh, 1995). In addition to conventional chemotherapies, ART was also combined with the immune modulatory drug LEN (Galustian & Dalgleish, 2009). These in vitro studies demonstrated the effects of ART on the cell cycle, and the studies showed restoration of cytotoxicity in an ART-resistant cell by adopting a pulsed-schedule of combination treatment. The mechanism underlying the combinatorial interaction, and indeed the mechanism of ART action in cancer per se is still not fully elucidated; however, those studies are ongoing and currently form the basis of further studies (Liu et al., 2011).

Many antiangiogenic and antivascular agents are now in clinical trials for the treatment of cancer. It is conceivable that loading tumor cells with bivalent iron by simply providing Fe^{2+} in tablet form might increase the susceptibility of cancer cells to the action of AS. It is tempting to speculate that, in the case of the second patient in the Berger study the addition of Fe^{2+} had an actual clinical impact and resulted in an improved response to therapy (Berger et al., 2005). Continued research in this area is encouraged by the recent success of a Phase II clinical trial of

AS combined with NP chemotherapy in treatment of advanced non-small cell lung cancer. The disease controlled rate of the trial group of AS plus NP chemotherapy (88.2%) was significantly higher than that of the NP chemotherapy alone group (72.7%), and the trial group's time to progression (24 weeks) was significantly longer than that of the NP chemotherapy alone group (20 weeks). AS combined with NP chemotherapy can increase the short-term survival rate of patients with advanced non-small cell lung cancer and prolong the time to progression without extra side effects (Zhang et al., 2008). The diversity in the targets of ART supports the possibility that it could be used in combination with other agents.

5.3 Toxicity avoidance strategies when employing ARTs

At high concentrations, ARTs appear to be active against cancer *in vivo*. However, the use of ARTs at high concentrations or for long drug exposure times has substantial risk of severe toxicities, both embryotoxicity and neurotoxicity. Animal data have shown that high concentrations of AS and DHA can induce embryotoxicity, and the longer exposure times associated with therapy using oil-soluble ARTs, such as artemether, will produce fatal neurotoxicity (Li et al., 2007a). To prevent embryotoxicity in pregnant women with malaria, current WHO policy recommendations on the use of ARTs in uncomplicated malaria state that ARTs should be used only in the second and third trimester, limiting the use of ARTS in the first trimester to cases where it is the only effective treatment available (WHO 2006b).

Studies with laboratory animals have demonstrated fatal neurotoxicity associated with intramuscular administration of artemether (AM) and arteether (AE) or oral administration of artelinic acid (AL). These effects suggest that the exposure time of artemisinins was extended in these studies due to the accumulation of drug in the bloodstream, and this accumulation, in turn, resulted in neurotoxicity. In one study (Li and Hickman, 2011), the drug exposure time with a neurotoxic outcome (neurotoxic exposure time) was evaluated as a predictor of neurotoxicity *in vivo*. The neurotoxic exposure time represents a total time spent above the lowest observed neurotoxic effect levels (LONEL) in plasma. The dose of AE required to induce minimal neurotoxicity requires a 2-3 fold longer exposure time in rhesus monkeys (179.5 hr) than in rats (67.1 hr) and dogs (113.2 hr) when using a daily dose of 6-12.5 mg/kg for 7-28 days, indicating that the safe dosing duration in monkeys should be longer than 7 days under this exposure. Oral AL treatment required much longer LONEL levels (8-fold longer) than intramuscular AE to induce neurotoxicity, suggesting that water-soluble artemisinins appear to be much safer than oil-soluble artemisinins. Due to the lower doses (2-4 mg/kg) used with current artemisinins and the more rare use of AE in treating humans, the exposure time is much shorter in humans. Therefore, the current regimen of 3-5 days dosing duration should be quite safe. Advances in our knowledge of artemisinin-induced neurotoxicity can help refine the treatment regimens used to treat malaria with oral ARTs as well as injectable AS products to avoid the risk of neurotoxicity. Although the water-soluble artemisinins, like AS, appear to be much safer, further study is needed in when employing ARTs as anticancer agents (Li & Hickman, 2011).

5.4 Strategies to utiliize ART derivatives as anticancer agents

As mentioned above, AS is completely converted to DHA and is best described as a prodrug of DHA. Also, DHA was shown to be more effective than AS in inhibition of angiogenesis and vasculogenesis *in vitro* (Longo et al., 2006a; 2006b; Chen et al., 2004; White et al., 2006). In addition, , the embryotoxicity and neurotoxicity of artemisinins can be reduced by using artemisone, which is a novel derivative of artemisinin that is not metabolized to DHA (Figure 1) (D'Alessandro et al., 2007; Schmuck et al., 2009).

Artemisone is a novel amino alkyl ART that has recently entered Phase II clinical trials (D'Alessandro et al., 2007). The compound was rationally designed to have reduced lipophilicity in order to impede transport to the brain and embryo. In addition, the inclusion of a thiomorpholine 1,1-dioxide group at the C10 position blocks the conversion of artemisone to the more lipophilic DHA. This structural modification does not affect anti-parasitic activity but reduces neurotoxicity and embryotoxicity, as assessed in primary neuronal brain stem cell cultures from fetal rats and in vivo in female rats (Schmuck et al., 2009). The retention of artemisone antimalarial activity infers that chemical activation of the peroxide bridge to a toxic parasiticidal chemical species remains unchanged, but recent literature also suggests that artemisone has a direct cytotoxic activity without activation of the endoperoxide bridge. In fact, two subsequent studies have provided conflicting results concerning the dependence of the pharmacological activity of artemisone on iron-activation of the endoperoxide group.

Interestingly, in an in vitro study by D'Alessandro et al, the anti-angiogenic effects of artemisone were reduced compared with DHA, and it was suggested that this reduction may limit the potential of artemisone to cause embryotoxicity mediated by defective angiogenesis and vasculogenesis during embryo development (D'Alessandro et al., 2007). Together these studies suggest that, while artemisone was designed to optimize safety by physicochemical means, the structural changes induced to create artemisone may also affect the intracellular chemical and molecular pathways which underlie toxicity, perhaps via reduced or alternative mechanisms of bio-activation and/or reduced cellular accumulation, when compared with the traditional ARTs. Therefore, artemisone represents an exciting novel compound in which increased anti-parasitic activity is combined with a reduced potential to cause both embryotoxicity and neurotoxicity.

Increased knowledge of the molecular mechanisms of ART-derived drugs and recent developments in novel ART applications demonstrates that further pharmacokinetic and pharmacodynamic analyses of novel ART derivatives are needed to understand why these compounds differ in efficacy and toxicity. This information will prove useful for the rationale design of more-effective ART-based molecules for use as anticancer agents. New derivatives of ARTs may act not only as treatment drugs, but also may have potential as potent cancer preventative agents due to their inhibition of tumor promotion and progression.

6. Conclusion

AS and its bioactive metabolite, DHA, have been the topic of considerable research study in recent years. Both drugs have been used to effectively treat infections with different forms of malarial parasites including multidrug-resistant strains. The key structural feature in all of the artemisinin (ART)-related molecules that mediates their antimalarial activity, and some of their anticancer activities, is an endoperoxide bridge. ARTs have been shown to induce apoptosis, a highly ordered form of parasite suicide, affecting both mature and immature parasites. Apoptosis is widely believed to be the mechanism by which ART therapy rapidly kills malaria parasites. However, severe embryotoxicity in a number of animal species has been reported after treatment with AS and DHA. A number of research studies have shown that the mechanism of ART embryotoxicity appears to be associated with ART- driven inhibition of fetal hematopoiesis and vasculogenesis. Specifically, higher drug levels of AS and DHA have been shown to affect erythroblasts, endothelial cells and cardiovascular cells in the early embryo. The inhibition of angiogenesis induced by ART-derived drugs has also been shown to be a mechanism of anticancer activity *in vitro* and *in vivo*. In particular, cancer angiogenesis plays a key role in the growth, invasion, and metastasis of tumors. ARTs-

induced inhibition of angiogenesis could be a promising therapeutic strategy for treatment of cancer. Other anticancer mechanisms induced by ARTs have been recognized recently that have guided various clinical trials in anticancer therapy. Since new and alternative vascularization mechanisms have been found, further research on the mechanism of efficacy and toxicity could lead us to understand more deeply the possibilities inherent in therapeutic development of ARTs for malaria, cancer, and other indications. The new therapeutic strategies for use of ARTs should be also considered to avoid problems associated with reproductive toxicity and neurotoxicity.

Taken together, the ARTs and the derivatives of ARTs have been shown to have potent antivasculogenic and antiangiogenic effects in tumor cells as well as in healthy embryos in animals and cultures. These observations have many implications in terms of cancer therapy and prevention as well as avoidance of drug toxicity associated with inhibition of vasculogenesis and angiogenesis.

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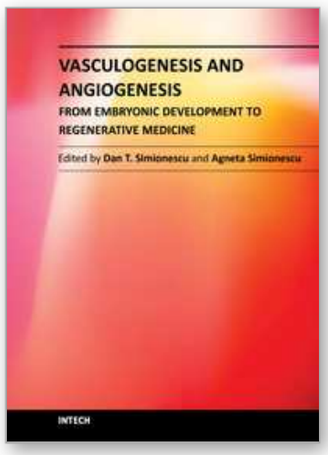

Vasculogenesis and Angiogenesis - from Embryonic Development to Regenerative Medicine

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Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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