

# Anti-angiogenic properties of artemisinin derivatives (Review)

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**Abstract.** Angiogenesis, the process involving the development of new blood vessels from existing capillaries, is critical for growth and wound healing. However, pathological angiogenesis contributes to the pathogenesis of numerous diseases, including cancer, rheumatoid arthritis, diabetic retinopathy and macular degeneration. Hence, the inhibition of angiogenesis is an effective therapeutic approach for these diseases. Apart from its anti-malarial properties, artemisinin and its derivatives also exhibit potent anti-angiogenic properties. The molecular mechanisms underlying their inhibitory effects on angiogenesis have been studied by several groups. These investigations have revealed that artemisinins inhibit angiogenesis via the perturbations of cellular signaling pathways involved in the regulation of angiogenesis. Along with a brief introduction to artemisinin derivatives, this review provides a detailed summary of

the effects of artemisinins on the mitogen-activated protein kinase (MAPK) pathway, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway. Due to the multiplicity of their actions on relevant signaling pathways, artemisinins are promising candidates with potential for use as anti-angiogenic agents for the treatment of related diseases or disorders.

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**Abbreviations:** 4E-BP1, eukaryotic translation initiation factor 4E binding protein; ANG, angiopoietin; AP-1, activator protein-1; ART, artesunate; Atf-2: activating transcription factor-2; COX-2, cyclooxygenase-2; DHA, dihydroartemisinin; EC, endothelial cell; eIF-4E, eukaryotic translation initiation factor 4E; ERK, extracellular signal-regulated kinase; HIF, hypoxia inducible factor; HUVEC, human umbilical vein endothelial cell; IL-8, interleukin-8; I $\kappa$ B, inhibitor  $\kappa$ B; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloprotease; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphatidylinositol 3-kinase; RA, rheumatoid arthritis; RAFLS, rheumatoid arthritis fibroblast-like synoviocytes; ROS, reactive oxygen species; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2

**Key words:** artemisinin, angiogenesis, mitogen-activated protein kinase pathway, Akt/phosphatidylinositol 3-kinase pathway, nuclear factor- $\kappa$ B pathway

## 1. Introduction

Angiogenesis refers to the process through which new blood vessels form from the pre-existing vasculature (1-8). It requires a complex interplay between angiogenic stimuli and angiogenic repressors, which leads to the controlled activation, proliferation, and migration of endothelial cells (ECs) (9-11). Normal angiogenesis plays a vital role in growth, development and wound healing (9,10). However, angiogenesis is also initiated in a number of diseases due to the imbalanced production of angiogenic regulators (12). Pathological angiogenesis results in an aberrant vasculature which accelerates disease progression (9,11). To elaborate, elevated levels of vascular endothelial growth factor (VEGF) are responsible for angiogenesis in cancer, a process that provides blood supply for tumor growth and metastasis (9,13-15). Likewise, the increased retinal expression of VEGF in response to hyperglycemic damage leads to ocular neovascularization which increases the risk of vision loss in diabetic retinopathy (16,17). Similar observations also present in rheumatoid arthritis (RA) and macular degeneration (9,14,18,19). In view of the pivotal role of angiogenesis in the pathogenesis of numerous diseases, the inhibition of angiogenesis by the use of anti-angiogenic agents has become an important therapeutic approach (20).

Artemisinin is extracted from the traditional Chinese medicine 'qinghao' (*Artemisia Annua* L.) (9,18). Its derivatives are

renowned for their potent anti-malarial effects and reliable safety records (9,18). During the past decade, emerging evidence has indicated that artemisinins also serve as effective treatments for cancer (9,13-15). The effectiveness of artemisinins in cancer at least in part relies on the inhibition of tumor angiogenesis (9,15). For example, the daily injection of dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, reduces the density of the tumor vasculature and consequently impairs tumor growth in mouse models (21). Moreover, other derivatives, such as artesunate (ART) and artemether display similar anti-angiogenic properties (15,18,22). Hence, artemisinins demonstrate auspicious potential for use as novel treatments for a wider variety of angiogenesis related diseases (23). There is evidence to suggest that several signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway, may mediate the inhibitory effect of artemisinin derivatives on angiogenesis (1,2,18,24,25). Beginning by describing the characteristics of artemisinin derivatives, this review provides a comprehensive explanation of the current literature regarding the molecular mechanisms underlying the effects of artemisinins on angiogenesis.

## 2. Derivatives of artemisinin and their characteristics

Following the isolation of artemisinin, the parent compound, semi-synthetic derivatives, such as artemether, arteether and ART have been developed with improved pharmacokinetics (26-28). The lipid-based derivatives, artemether and arteether, are highly lipophilic (15,29). While possessing longer half-lives than more hydrophilic artemisinins, both compounds are better at transpassing the blood-brain barrier (15,29). Coupled with their anticancer activity, they may be exceptionally efficient in treating brain tumors (15,29). Due to the addition of a hemisuccinate group, out of all the artemisinins, ART has the best water solubility and bioavailability (9). Experiments such as human umbilical vein endothelial cell (HUVEC) migration assays have confirmed that ART successfully inhibits angiogenesis induced by human melanoma cells with a much lower concentration (3,30). Nevertheless, artemisone and artemiside, two relatively newer 10-alkylaminoartemisinin derivatives, seem to have superior efficacy compared to ART (31,32). Although artemiside still has to undergo a toxicological evaluation, it has been well-established that artemisone has negligible toxicity, particularly neurotoxicity owing to its low lipophilicity (31-33).

Despite the variability in the structure and biological properties of artemisinin derivatives, all compounds are metabolized into DHA after being administered into the body (9). DHA can also be synthesized artificially and possesses additional water solubility; hence, it is the most studied artemisinin analog other than ART (3,9). On the other hand, novel derivatives of artemisinins are constantly being developed with further refined pharmacological properties (34). As argued by Jung *et al*, non-acetal-based artemisinins have lower neurotoxicity than the above-mentioned acetal-based artemisinins (34). For example, dexoartemisinin- $C_{60}$  conjugate, which comprises of a dexoartemisinin dimer and a fullerene cage demonstrates potent anti-angiogenic activity in chorioallantoic membrane assay with enhanced efficacy and expected lower toxicity (34).

## 3. Mechanisms underlying the anti-angiogenic effects of artemisinin derivatives

*The MAPK pathway.* MAPKs, encompassing extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and p38 MAPK, are involved in a wide range of cellular activities (1,35). ERKs, which regulate cell proliferation and survival, can be activated by downstream signals of VEGF (1). The binding of VEGF with its receptors on ECs stimulates a conformational change of the Ras protein, which subsequently leads to the phosphorylation of Raf (1). Activated Raf in turn phosphorylates MEK1/2, direct activators of ERKs (1). This cascade of signals eventually results in the promotion of EC proliferation and survival (1). Unlike ERK, JNK and p38 MAPK mediate both cytoprotective and cytotoxic processes (35-37). JNK is a pro-angiogenic protein which can be induced by cellular stressors, such as hypoxia or inflammation (35,36). Upon activation, JNK phosphorylates the c-jun component of the activator protein-1 (AP-1), which results in the nuclear translocation of both c-jun and activating-transcription factor-2 (Atf-2) (36,38). Consequently, the expression levels of pro-angiogenic stimuli, including VEGF, cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs) are increased (20,36,38-41). p38 MAPK responds to stress-related extracellular stimuli in a similar manner (37). On the other hand, both JNK and p38 MAPK are key mediators of apoptosis (11,42,43). It has been hypothesized that JNK is critical for the activation of pro-apoptotic protein Bax (43). In addition to Bax, p38 MAPK also upregulates the pro-apoptotic Fas, while inhibiting proteins that promote cell survival (ERK and Akt) (37).

The artemisinin family drugs act upon MAPK signaling cascades in multiple ways. DHA inhibits HUVEC proliferation by blocking both the transcription and activation of ERK1/2 (1). The incubation of HUVECs with DHA (20  $\mu$ M) for up to 12 h was shown to successfully reduce the expression of ERK1/2 at both the mRNA and protein level (1). Together with the decreased level of phosphorylated ERK1/2, these results were accompanied by a dose-dependent decrease in HUVEC proliferation (1) (Fig. 1). Moreover, the addition of PD98059, an inhibitor of MEK1/2, resulted in a comparable inhibition of HUVEC proliferation (1). Furthermore, the co-administration of DHA and PD98059 did not lead to further reduction in the proportion of proliferating HUVECs (1). Since ERK1/2 are activated by MEK1/2 only, the lack of additive effect between PD98059 and DHA justifies that DHA restrains angiogenesis by impeding ERK related cytoprotective activities (1) (Table I).

As previously demonstrated, DHA, at a concentration of 20  $\mu$ M, significantly increased the level of activated JNK in HUVECs at 6 h of incubation (44). In addition, the level of activated JNK plateaued at 12 h-incubation before starting to decline at 24 h (44). Intriguingly, although the activation of JNK is also involved in apoptosis, DHA exerted no effect on HUVEC viability (44). Nonetheless, beginning from a concentration of 12.5  $\mu$ M, ART decreased the level of activated JNK in HUVECs following incubation for 0.5 h (11) (Fig. 1). Accordingly, the proliferation of ART-treated HUVECs was also inhibited (11). The results from these two studies rise controversy regarding the distinctions in effects of different artemisinin analogs on JNK activation (Table I).

Table I. Mechanisms underlying the anti-angiogenesis effects of artemisinin derivatives.

<i>In vitro</i> experiments				
Analog	Cell type	Effect	Mechanism	(Refs.)
Experiments on ECs				
ART	HUVECs	Proliferation ↓	JNK activation ↓	(11)
		Apoptosis ↑	p38 MAPK activation ↑	(11)
DHA	HUVECs	Proliferation ↓	ERK signalling ↓	(1)
		Migration ↓	Independent of p38 MAPK activation	(3)
		Proliferation and migration ↓	VEGFR2 expression ↓	(2)
			Nuclear translocation and DNA binding capacity of NF-κB ↓	(2,21)
Experiments on non-ECs				
ART	RAFLS	Production of VEGF and IL-8 ↓	Akt phosphorylation ↓	(18)
		IL-8 production ↓	Nuclear translocation and DNA binding capacity of NF-κB ↓	(52)
			Akt phosphorylation ↓	(52)
DHA	Rhabdomyosarcoma cells Ewing sarcoma cells	VEGF production ↓	Blockade of mTORC1	(24)
<i>In vivo</i> experiments				
Analog	Animal model	Effect	Mechanism	(Refs.)
ART	Sprague-Dawley rats	Corneal neovascularization ↓	p38 MAPK activation ↑	(11)
DHA	BALB/c nude mice	Production of pro-angiogenic cytokines ↓	NF-κB activity ↓	(21)
	C57BL/6N mice	Tumor microvessel density ↓		
		Retinal neovascularization ↓	NF-κB activity ↓	(2)

EC, endothelial cell; ART, artesunate; HUVECs, human umbilical vein endothelial cells; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; DHA, dihydroartemisinin; ERK, extracellular signal-regulated kinase; VEGFR2, vascular endothelial growth factor receptor 2; NF-κB, nuclear factor-κB; RAFLS, rheumatoid arthritis fibroblast-like synoviocytes; VEGF, vascular endothelial growth factor; IL, interleukin; mTORC1, mammalian target of rapamycin complex 1. The upward arrows indicate the increase in the activities, and the downward arrows indicate the decrease in the activities.

In a previous study, compared to the phosphate-buffered saline (PBS)-treated controls, ART significantly increased the proportion of apoptotic HUVECs by inducing p38 MAPK activation (11). Further investigation revealed that activated p38 MAPK leads to an increase in the Bax/Bcl-2 ratio and the cleavage of caspase-9, which ultimately results in apoptosis via the intrinsic mitochondrial pathway (11) (Fig. 1). Moreover, pre-treatment of HUVECs with a p38 MAPK inhibitor (SB203850) abolished the ART-induced activation of p38 MAPK, while it decreased the proportion of apoptotic cells (11) (Table I). Treatment with ART (25 μM) was also able to reduce rat corneal neovascularization in response to alkaline burns (11). In addition, TUNEL and CD31 double staining of those corneal sections revealed a substantially larger proportion of apoptotic vascular ECs in the ART-treated group (11) (Table I). Therefore, both *in vitro* and *in vivo* experiments suggest that ART inhibits angiogenesis by activating p38 MAPK and promoting EC apoptosis (11). Notably, the pro-apoptotic effect of ART seems to rely on the formation of reactive oxygen species (ROS) via the cleavage of the endoperoxide bond by

ferrous iron (9,11,45,46). The reduced phosphorylation of p38 MAPK was observed simultaneously with the inhibition of ROS generation, whereas the addition of ferrous iron along with ART facilitated ROS production and increased the proportion of apoptotic ECs (11). Intriguingly, although the possession of an endoperoxide bond is a common feature of all artemisinin derivatives, DHA (20 μM) restricts EC proliferation and migration without inducing apoptosis (1,3,15). Seeing the role of p38 MAPK in ART-induced EC apoptosis, such a result provides little evidence for DHA to have a comparable influence to ART on p38 MAPK signaling (11). Indeed, DHA (20 μM) did not induce any change in the level of either p38 MAPK or activated p38 MAPK in HUVECs (3) (Fig. 1). Moreover, the blockade of p38 MAPK by SB203850 had no effect on the DHA-suppressed EC migration (3) (Table I). Therefore, unlike ART, DHA inhibits EC migration via a mechanism that is independent of p38 MAPK.

**The NF-κB pathway.** In addition to its role in innate immunity, NF-κB regulates the transcription of numerous angiogen-

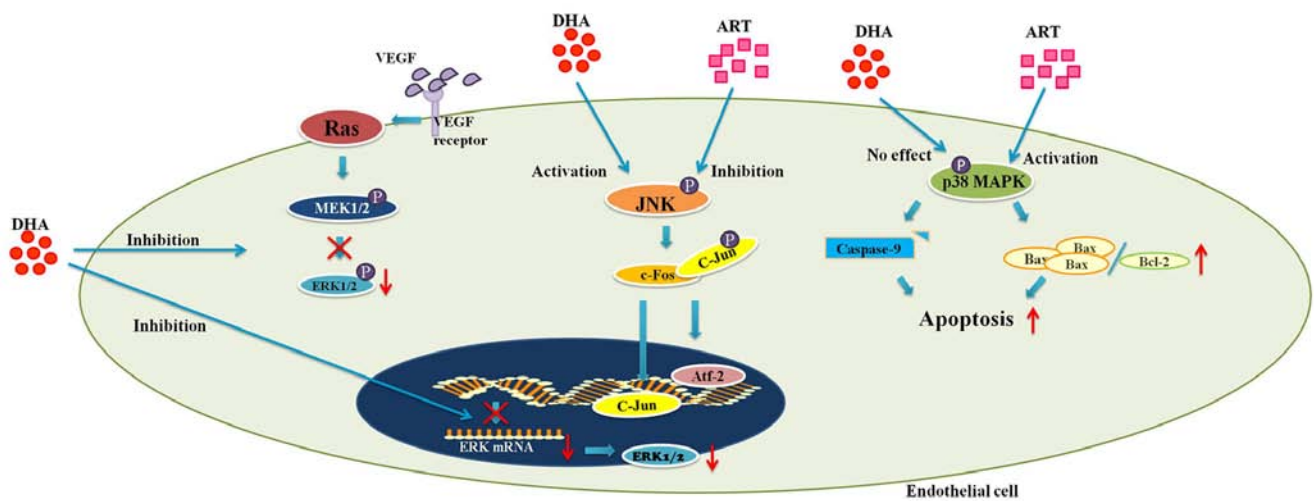


Figure 1. Effects of artemisinins on the mitogen-activated protein kinase (MAPK) cascades in endothelial cells.

esis-related genes (47). For instance, in hypoxia-induced angiogenesis, hypoxia inducible factor- $\alpha$  (HIF- $\alpha$ ) becomes stabilized owing to the inhibition of prolyl hydroxylase-1, which leads to increased degradation of inhibitor  $\kappa$ B (I $\kappa$ B) (48). Since I $\kappa$ B is responsible for the masking of NF- $\kappa$ B (p65) nuclear localization sequence, its degradation results in the increased nuclear translocation of NF- $\kappa$ B and subsequently increased transcriptional expression of vascular endothelial growth factor receptor 2 (VEGFR2) (2,48). VEGFR2 is crucial in mediating the VEGF-induced activation of pro-angiogenic signaling pathways (40). Hence, NF- $\kappa$ B plays an irreplaceable role in angiogenesis by regulating the production of VEGFR2 (2,40).

On the other hand, the activation of NF- $\kappa$ B by other pro-inflammatory factors, such as tumor necrosis factor (TNF) may induce HIF-1 $\alpha$  under normoxic conditions (41). Indeed, many inflammation-related cytokines and chemokines modulated by NF- $\kappa$ B are pro-angiogenic factors (41). For example, *in vitro* experiments with HUVECs have shown that interleukin-8 (IL-8) promotes tube formation as well as EC infiltration (49). Similarly, the incubation of human appendix ECs with various chemokines has been shown to result in the formation of pseudovessels (50). Additionally, the activation of NF- $\kappa$ B by lipopolysaccharides may directly upregulate HIF-2 and thus induce an increase in nitric oxide (NO) production (41). NO at a high concentration ( $>1 \mu$ M) in turn stabilizes HIF- $\alpha$  expression and consequently increases the production of both VEGF and its receptors (41,51).

There is extensive evidence to suggest that interactions between artemisinins and NF- $\kappa$ B signaling inhibit angiogenesis (2,15,21). In particular, DHA was found to prevent the nuclear translocation of NF- $\kappa$ B in HUVECs by increasing the I $\kappa$ B level (2). Consequently, the production of VEGFR2 was decreased (2) (Fig. 2). Moreover, DHA downregulates the binding of NF- $\kappa$ B p65 to the promoter region of VEGFR2 (2). The lack of synergy between DHA and a known NF- $\kappa$ B inhibitor [pyrrolidine dithiocarbamate (PDTC)] further confirms that DHA operates by interfering with the NF- $\kappa$ B pathway (2). Considering the aforementioned role of VEGFR2, suppressed VEGFR2 production conceivably explains the reduced EC proliferation and migration following treatment

with DHA (2). Moreover, the daily injection of DHA into the vitreous humor substantially reduced retinal neovascularization in mice models (2). Moreover, combined treatment with DHA and PDTC resulted in no further reduction in retinal vessel density (2) (Table I). Therefore, DHA inhibits angiogenesis *in vitro* and *in vivo* by blocking NF- $\kappa$ B signaling (2).

In the meantime, artemisinins exert their anti-angiogenic effects by ameliorating NF- $\kappa$ B-mediated inflammation (9,21,52). Pretreating human RA fibroblast-like synoviocytes (RAFLS) with ART (1  $\mu$ M) significantly suppressed NF- $\kappa$ B mediated IL-8 production induced by TNF- $\alpha$  (52). Following the addition of TNF- $\alpha$ , ART prevented I $\kappa$ B degradation, leading to the reduced nuclear translocation and weakened DNA-binding capacity of NF- $\kappa$ B (52). *In vitro* experiments using HUVECs treated with DHA produced almost identical results (21) (Table I). Since IL-8 has long been recognized as a pro-angiogenic cytokine, it appears that artemisinins may inhibit angiogenesis by interfering with NF- $\kappa$ B signaling and consequently inhibiting IL-8 production (49). In addition, the daily injection of DHA into mice with xenografts of the pancreatic cancer cell line, BxPC-3, was shown to result in a dose-dependent reduction of VEGF, IL-8 and COX-2 in tumor cells (21). Moreover, the reduced production of the above-mentioned pro-angiogenic cytokines was accompanied by reduced NF- $\kappa$ B activity and decreased tumor microvessel density (21) (Fig. 2) (Table I). Taken together, artemisinins inhibit angiogenesis by suppressing the secretion of NF- $\kappa$ B regulated pro-angiogenic cytokines (21,49).

**The PI3K/Akt/mTOR pathway.** The role of PI3K and its downstream targets Akt/mTOR in angiogenesis involves the modulation of VEGF expression and other angiogenic stimuli such as NO and angiopoietins (ANGs) (53). In mammals, PI3K regulates the expression of mTOR which phosphorylates the eukaryotic translation initiation factor 4E binding protein (4E-BP1) (54). The phosphorylation of 4E-BP1 reduces the stability of a complex consisting of eukaryotic translation initiation factor 4E (eIF-4E) and 4E-BP1 (54). Since the eIF-4E/4E-BP1 complex inhibits HIF-1 $\alpha$  translation, phosphorylated mTOR leads to 4E-BP1 activation which increases

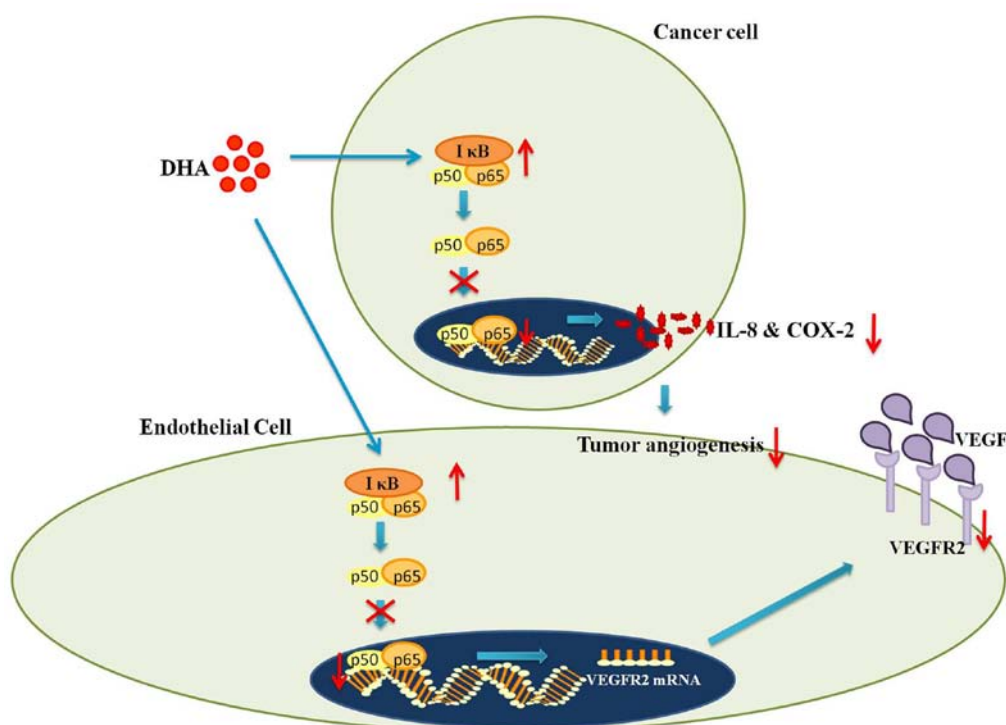


Figure 2. Anti-angiogenic effect of dihydroartemisinin (DHA) via perturbation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway.

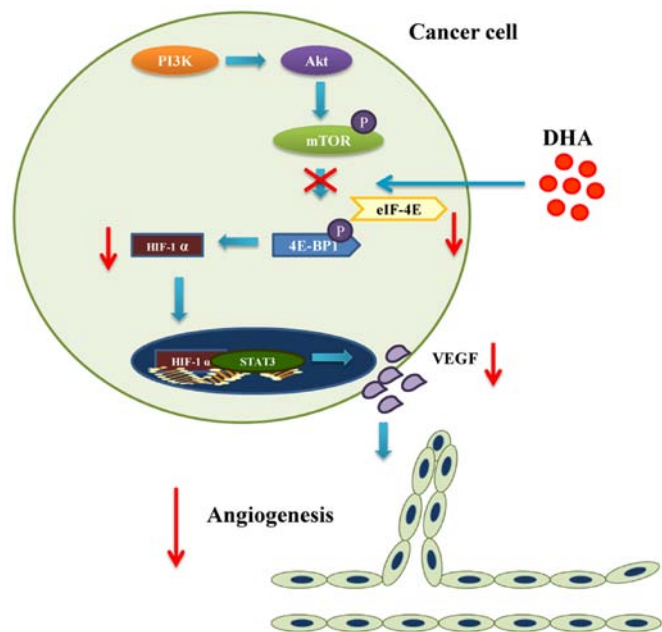


Figure 3. Predicted effect of dihydroartemisinin (DHA) on tumor angiogenesis mediated by the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway.

the expression of HIF-1 $\alpha$  (54). In addition, Akt is able to activate endothelial NO synthase (eNOS), one of the regulators of NO synthesis in tumors (53). Activated eNOS mediates VEGF induced EC migration (53). Meanwhile, ANGs and their receptors are another class of growth factors facilitating the effect of VEGF that are related to the PI3K/Akt/mTOR pathway (53).

ART inhibits angiogenesis by preventing Akt activation. ART reduces the production of pro-inflammatory cytokines

and VEGF in human RAFLS (18). To elaborate, PI3K inhibitor inhibits the production of several pro-inflammatory cytokines including the pro-angiogenic IL-8 (52). The inhibition of PI3K also correlates with reduced expression and nuclear translocation of HIF-1 $\alpha$  (18). Accordingly, the transcriptional expression of VEGF is decreased (18). There is evidence to suggest that ART prevents Akt phosphorylation, while hampering the production of VEGF and IL-8 in a similar manner (18,52) (Table I). Apart from IL-8 and VEGF, the decreased phosphorylation of Akt is likely to diminish the effect of eNOS and ANG2 on angiogenesis. Therefore, inhibited Akt activation by ART leads to reduced production of angiogenic stimuli (18,52).

Results from numerous studies have indicated that DHA also functions as a PI3K/Akt/mTOR inhibitor (55-57). Apart from inhibiting Akt activity, DHA primarily exerts its effect by interacting with mTOR (24,58). DHA effectively blocks mTOR complex 1 (mTORC1) in rhabdomyosarcoma cells and Ewing sarcoma cells in both a dose- and time-dependent manner (24,25). As a result, binding between 4E-BP1 and eIF-4E is enhanced (24). Since tumor angiogenesis is powered by the sustained secretion of VEGF by tumor cells under hypoxic stress, it relies on the stabilization of HIF-1 $\alpha$  induced by degradation of 4E-BP1 eIF-4E complex (54). Blockade of mTORC1 by DHA impairs the ability of tumor cells to secrete VEGF, which arguably contributes to the inhibition of tumor angiogenesis (Fig. 3 and Table I).

The effects of artemisinins on selected signaling pathways may depend on cell types. For example, as previously demonstrated, ART activated none of the three MAPKs in TNF- $\alpha$  stimulated RAFLSs, which is in contrast with the findings using HUVECs (11,18,52). Likewise, DHA inhibited ERK signaling in HUVECs but did not alter ERK signaling in cultured T cells (1,55). Overall, ECs seem to be especially susceptible to

influences of artemisinins, which again signifies the potential for artemisinin derivatives to be used as anti-angiogenic agents. Moreover, the results mentioned above suggest that artemisinin derivatives may have distinct actions in different disease models. Hence, tailoring treatment schemes according to these variations may optimize the outcome.

#### 4. Conclusion

Apart from anti-malaria, extensive evidence suggests that artemisinins inhibit angiogenesis. The effects of artemisinins on angiogenesis rely on perturbations of MAPK pathway, NF- $\kappa$ B pathway, and PI3K/Akt/mTOR pathway (1,2,11,21,52). DHA inhibits EC proliferation by reducing ERK1/2 expression and activation (1,11). In the meantime, ART and DHA appear to play distinct roles in JNK and p38 MAPK activation (3,11,44). In addition to decreasing VEGFR2 expression in ECs, artemisinins limit angiogenesis by mitigating the production of pro-angiogenic cytokines from tumor cells (2,21,52). Both actions are achieved by the inhibition of NF- $\kappa$ B activity (2,21,52). Furthermore, since artemisinins prevent activation of both Akt and mTOR, they are able to interfere with relevant downstream pro-angiogenic gene transcription to inhibit angiogenesis (24,25,52,53,55-59). The pleiotropy of the effects of artemisinins renders them as potent anti-angiogenic agents. In view of the significance of angiogenesis in pathogenesis of many diseases, artemisinin and its derivatives are excellent candidates to be used in novel therapies (18).

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