


REVIEW ARTICLE

Artemisinin as an anticancer drug: Recent advances in target profiling and mechanisms of action

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Abstract

Artemisinin and its derivatives (collectively termed as artemisinins) are among the most important and effective antimalarial drugs, with proven safety and efficacy in clinical use. Beyond their antimalarial effects, artemisinins have also been shown to possess selective anticancer properties, demonstrating cytotoxic effects against a wide range of cancer types both *in vitro* and *in vivo*. These effects appear to be mediated by artemisinin-induced changes in multiple signaling pathways, interfering simultaneously with multiple hallmarks of cancer. Great strides have been taken to characterize these pathways and to reveal their anticancer mechanisms of action of artemisinin. Moreover, encouraging data have also been obtained from a limited number of clinical trials to support their anticancer property. However, there are several key gaps in knowledge that continue to serve as significant barriers to the repurposing of artemisinins as effective anticancer agents. This review focuses on important and emerging aspects of this field, highlighting breakthroughs in unresolved questions as well as novel techniques and approaches that have been taken in recent studies. We discuss the mechanism of artemisinin

Abbreviations: ABC, ATP-binding cassette; ALA, α -aminolevulinic acid; BDHA, biotinylated dihydroartemisinin; CQ, chloroquine; CSC, cancer stem cell; DFO, desferrioxamine; DHA, dihydroartemisinin; DJ-1, PARK7/Parkinson disease protein 7; ER, endoplasmic reticulum; HNSCC, head and neck squamous cell carcinoma; HSP, heat-shock protein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MS, mass spectrometry; mTOR, mammalian target of rapamycin; NSCLC, nonsmall cell lung cancer; PFDHA, 10 β -(*p*-fluorophenoxy) dihydroartemisinin; PGE₂, prostaglandin E₂; ROS, reactive oxygen species; SA, succinyl acetone; STAT3, signal transducer and activator of transcription 3; TfR1, transferrin receptor 1; TPP, triphenyl phosphonium; Treg, regulatory T cell

activation in cancer, novel and significant findings with regards to artemisinin target proteins and pathways, new understandings in artemisinin-induced cell death mechanisms, as well as the practical issues of repurposing artemisinin. We believe these will be important topics in realizing the potential of artemisinin and its derivatives as safe and potent anticancer agents.

KEYWORDS

artemisinin, anticancer, targets identification, mechanism of action, chemical proteomics

1 | INTRODUCTION

Artemisinin and its derivatives (collectively termed as artemisinins) are sesquiterpene lactones derived from the sweet wormwood (*Artemisia annua*), which has been used in Chinese traditional medicine for thousands of years as a remedy for fevers and chills.¹ Following their discovery and development as antimalarial drugs by Tu Youyou's group in the 1970s, artemisinin (**1**) and its semisynthetic derivatives including artesunate (**2**), artemether (**3**), and their common active metabolite dihydroartemisinin (DHA) (**4**) have come to represent the current front line in antimalarial medicine²⁻⁵ (Fig. 1). While the efficacy and low toxicity of the artemisinin compounds in the treatment of malaria is well-established, they have also been known to exhibit a range of beneficial effects in other capacities. These include antiviral and fungicidal properties, therapeutic functions in nonmalaria parasitic diseases, anti-inflammatory and anti-asthma effects as well as potential anticancer functions.⁶⁻¹³ Considering its status as an established and well-tolerated drug, it is naturally of interest to explore the repurposing of artemisinin for the treatment of nonparasitic diseases.¹⁴

Since the first report of the possible anticancer properties of artemisinin in 1993, a large volume of studies have highlighted the potential of artemisinin as a novel therapeutic agent for cancer.^{6,15,16} It is now understood that artemisinin and its derivatives exhibit highly selective and marked cytotoxicity against a range of cancer cell types both in vitro and in vivo, and great strides have been taken to unravel the mechanisms behind these effects.⁷ These studies have been covered by a good number of excellent reviews.^{12,14,17-20} The recent years have seen a surge in interest for artemisinin following the awarding of the Nobel Prize for Tu's discovery and efforts.²¹ It is apparent however that despite the volume of research available, several key issues remain under contention. First, the specificity of artemisinin-mediated cytotoxicity toward cancer cells has not been fully understood²². The mechanism of this specificity is likely related to the activation mechanism of artemisinin in cancer cells, which is similarly a debatable issue.¹⁹ Second, while there is a large body of evidence detailing the effects of artemisinin on numerous signaling pathways and cellular functions, a global view of its mechanism of action remains elusive. This review thus seeks to serve as a timely overview of some latest findings related to these issues as well as the novel techniques and approaches taken. Key mechanistic findings regarding drug activation and specificity, the protein targets of artemisinin and the mechanisms of cell death will be discussed, and other relevant considerations for the repurposing of artemisinin as an anticancer drug will be briefly highlighted.

2 | IRON AND HEME IN ACTIVATION AND ACTIVITY OF ARTEMISININ

Artemisinins are prodrugs that are activated under intracellular or intraparasitic conditions.^{22,23} The endoperoxide moiety of the artemisinins is known to be crucial both to its antiparasitic and anticancer properties, and artemisinin activation is often discussed based on the fact that this endoperoxide bridge can be cleaved to generate highly reactive carbon-centered radicals that can then participate in the alkylation of proteins and generate reactive oxygen

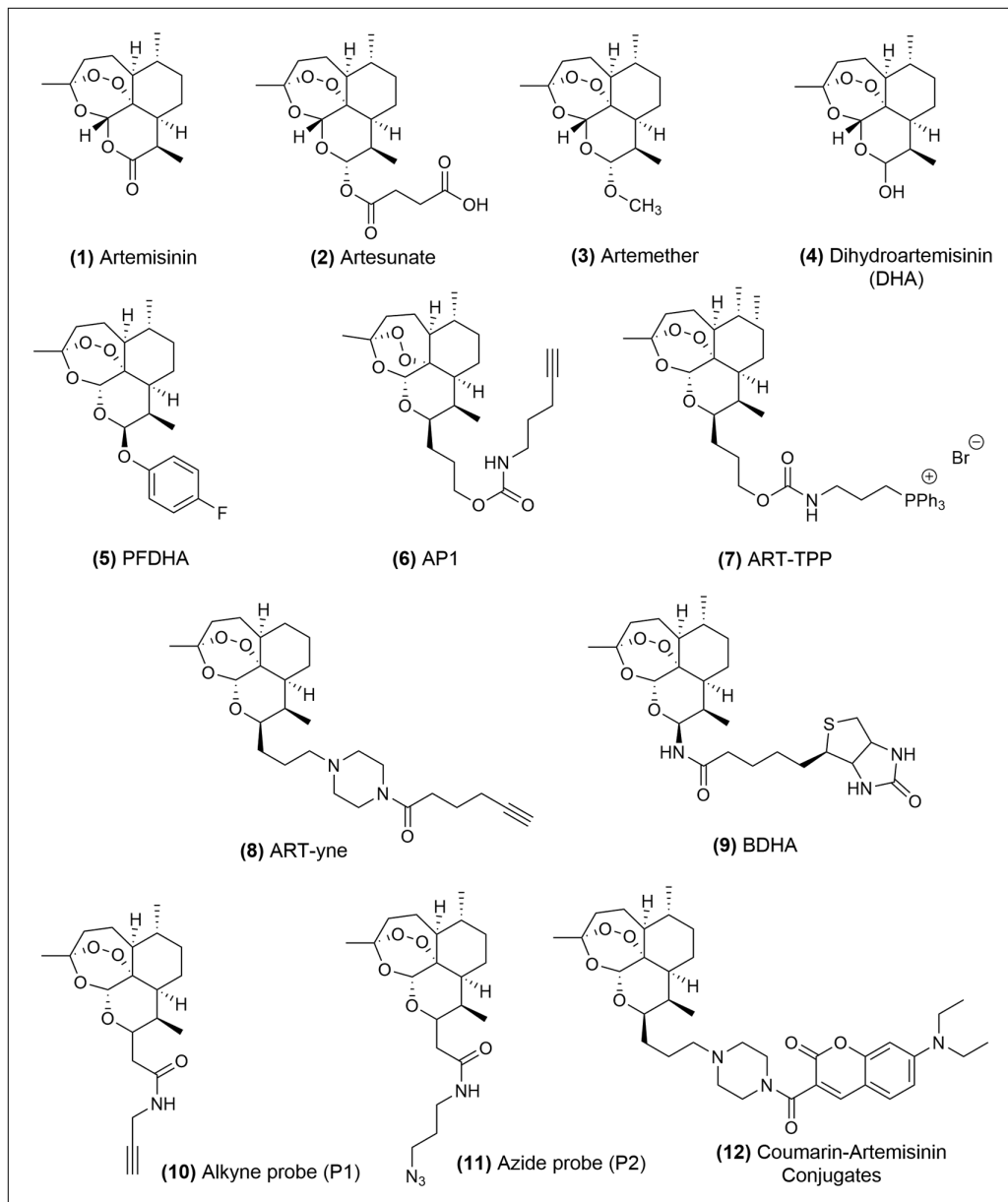


FIGURE 1 Chemical structures of artemisinin and its derivatives as well as modified artemisinin-based compounds referenced in this review. The numbering in the figure corresponds to bracketed numbers in the text

species (ROS).²⁴ What exactly mediates this cleavage and activation however remains a point of controversy, specifically regarding the involvement of free ferrous iron versus heme.²²

2.1 | Iron in artemisinin activation and activity

One prevailing hypothesis proposes free ferrous iron (Fe^{2+}) as the principal activator of artemisinin.^{14,18,19} This is an attractive viewpoint due to several reasons. First, the role of iron in artemisinin-mediated cytotoxicity is well-established. Many studies have shown that free cellular iron is directly linked to the anticancer properties of

artemisinin in a range of cell lines, and the chelation of free iron (frequently using desferrioxamine [DFO]) has been reported to almost completely abolish the effects of artemisinin.^{25–27} Cellular iron uptake and metabolism in terms of TfR1/CD71 (transferrin receptor 1) and ABC (ATP-binding cassette) transporter expression have also been linked to the efficacy of artemisinin, and the delivery of artemisinin in conjugate form with transferrin or holotransferrin (serum iron transport proteins) have been shown to greatly improve its effectiveness.^{25–30} Second, cancer cells are known to have higher requirements for iron metabolism and hence a higher cellular iron content compared to normal cells.³¹ This provides a highly plausible explanation for the cancer-specific nature of artemisinin, where the iron-rich environment of cancer cells results in enhanced artemisinin activation.

2.2 | Heme in artemisinin activation and activity

On the other hand, our understanding of the mechanism of artemisinin in its antimalarial function provides an alternative, heme-centric hypothesis for artemisinin activation. In malarial *Plasmodium* parasites where artemisinin is known to have remarkable efficacy, excessive parasitic digestion of hemoglobin results in heme release and a high-heme environment.^{32,33} Heme has also been reported to be a more potent activator of artemisinin compared to iron in parasites,^{34,35} supporting the notion that heme may serve as the primary activator rather than free ferrous iron. Importantly, a central role for heme in the activation and anticancer potential of artemisinin has also been reported. Work by Gerhard and colleagues provided strong evidence for the importance of heme in determining artemisinin activity through the manipulation of heme synthesis in cancer.³⁶ The addition of heme synthesis precursors increased the cytotoxicity of artemisinin while the heme synthesis inhibitor succinyl acetone (SA) had the opposite effect. Likewise, Stockwin et al. demonstrated a similar effect manipulating heme oxygenase (HMOX), a degradative enzyme of heme.³⁷ In their study, induction of HMOX inhibited the activity of artemisinin dimers and vice versa. Finally, Gerhard's team also monitored ROS production following artemisinin treatment using fluorescent probes and demonstrated a positive correlation between heme synthesis and ROS production as well as cytotoxicity.³⁸

Several studies have made use of mass spectrometry (MS) and chemical proteomics methods to probe the activity of artemisinin in a more direct manner. Mercer et al. first made use of MS to directly quantify the activation of PFDHA (10 β -*p*-fluorophenoxy) dihydroartemisinin) (5), a synthetic artemisinin derivative.³⁹ By comparing the amounts of remaining parent material following PFDHA cell treatment in the presence of heme synthesis modulators, it was found that inhibition of heme synthesis by SA almost completely abolished PFDHA activation. Recently, our group has taken the chemical proteomics approach, which employs chemically synthesized artemisinin-based probes to directly monitor the activity and binding behavior of artemisinin. In our study, we developed an artemisinin-based, alkyne-linked probe AP1 (6) that mimics the bioactivity of artemisinin while allowing click chemistry-based ligation with azide-linked fluorescent or affinity tags.⁴⁰ Upon activation, AP1 labels its targeted proteins that are then tagged with reporter groups via the AP1 alkyne handle. This approach thus enables both visualization and purification of artemisinin-binding proteins, as well as the direct observation of probe activation status under different conditions. In HCT116 colon cancer cells, the addition of the heme synthesis precursor α -aminolevulinic acid (ALA) significantly enhanced AP1 binding to cancer proteins while inhibition of heme synthesis had the opposite effect. In contrast, the addition of free iron (FeSO₄) had no significant effect on probe activation. We replicated this finding in a follow-up study, where the addition of hemin (heme containing ferric instead of ferrous iron) as a heme source resulted in probe activation in HCT116 cell lysates while FeSO₄ failed to achieve the same.⁴¹ Consistently, no probe activation could be observed in the absence of hemin, highlighting the importance of heme in artemisinin activation. Moreover, live HCT116 cells treated with the artemisinin probe had significantly higher probe activation and cytotoxicity with the supply of hemin, in comparison to those supplied with free iron. Crucially, the addition of the iron chelator DFO to hemin-treated cell lysates failed to abrogate probe activation, further supporting a principal role of heme, but not iron, as the key activator of artemisinin. Considering that mitochondria are the site of cellular heme synthesis, we then developed a mitochondria-targeting artemisinin probe ART-TPP (7), which showed a remarkable increase in cytotoxicity compared to artemisinin, demonstrating the practical implications of identifying the exact activating mechanism. Independently, Zhou et al. developed an alkyne-linked artemisinin probe ART-yne (8) and

carried out an in-depth mechanical analysis of probe activation in recombinant proteins as well as in HeLa lysates.⁴² Using MS detection of probe-hemin adducts under different conditions as a readout of probe activation, the group arrived at a similar conclusion that heme acts as the principal endoperoxide activator in artemisinin. Further details of the above-mentioned studies using the chemical proteomics approach will be discussed later in this review.

Based on the evidence discussed above, it may be prudent to consider a reexamination of the current understanding. There is sufficient evidence to consider a separation between drug activation and drug activity if we consider that the rescue of artemisinin-induced cytotoxicity by iron chelation (e.g., by DFO) need not be acting on the level of drug activation if downstream cellular effects are also iron-dependent. Heme-centric activation and iron-dependent cytotoxicity is thus a possible point of reconciliation between the existing points of view. It is also important to consider that clarifying the mechanism of action can lead to a greater understanding of the specificity of artemisinin to cancer, which has practical implications in therapy. Indeed, in addition to higher levels of cellular iron, cancer cells are also known to exhibit elevated levels of heme metabolism and it may be interesting to explore the potential link between the intrinsic capacity for heme synthesis and artemisinin efficacy in cancer.^{43–45} Finally, it may be useful to keep in consideration the role of alternative, endoperoxide-independent mechanisms of artemisinin, which may also contribute to its anticancer effects. While the primary mode of action of artemisinin is well understood to be endoperoxide-dependent, there is some evidence indicating the presence of residual endoperoxide-independent activity that may affect the interpretation of drug activation studies.^{18,46,47}

3 | TARGET AND PATHWAY IDENTIFICATION BY PROTEOMICS

Following activation by endoperoxide cleavage and subsequent generation of carbon-centered radicals, artemisinins are able to alkylate cellular proteins as well as generate ROS such as superoxide.^{7,22,24,39} A combination of oxidative stress, DNA damage, and other consequences of protein alkylation and disruptions to cellular functions then results in cell death.^{20,48–50} Apart from cell death, artemisinin is known to affect multiple hallmarks of cancer by inducing cell cycle arrest, inhibiting angiogenesis and impeding cancer metastasis and invasion.^{12,20} Much of the evidence and mechanisms behind these processes and the identities of the signaling pathways involved have been comprehensively reviewed and will not be repeated here.^{14,17,19,20,22} Instead, we will first highlight some recent studies in the use of emerging proteomics techniques to explore the complexities behind the anticancer effects of artemisinin. We will then discuss several recent and novel findings with regards to cellular targets and functions that may prove useful for future research.

Artemisinin has been linked to a wide range of cellular targets and pathways in cancer, with commonly reported representatives including the p38-MAPK, PI3K/Akt, Ras, NF- κ B, and Wnt/ β -catenin pathways, among others, which have been reviewed comprehensively in previous publications.^{12,14,18,20} Considering its general mechanism of protein alkylation and the range of reported effects, it is reasonable to suggest that artemisinin functions in a promiscuous or multitarget manner rather than acting on specific cellular targets. However, factors such as intracellular localization, level of activation, and the composition of the cellular proteome per se can all result in differential binding and hence varying effects in different cell types and conditions. In view of this multitargeting and diverse nature of artemisinin, it is increasingly apparent that high-throughput, discovery-based methods are necessary to complement classic pathway-centric approaches. Screening studies based on cell line panels and microarrays have been in use for some time and have led to important discoveries in identifying novel mediators for artemisinin efficacy and resistance.^{6,51,52} Visual methods using fluorescent probes of artemisinin have also been developed such as in the study by Liu et al. to investigate the intracellular localization of the compound, identifying the endoplasmic reticulum (ER) as the main site of localization.⁵³ In recent years however, new techniques and approaches have been emerging that allow for accelerated and direct profiling of implicated pathways and protein targets.

Advancements in MS techniques have enabled high-throughput, high-sensitivity, and quantitative proteomics analysis.⁵⁴ Accordingly, several recent studies, including our own, have made use of MS-based proteomics to directly study the mechanism of artemisinin in cancer at the proteome level. In a series of early studies, Lu et al. performed

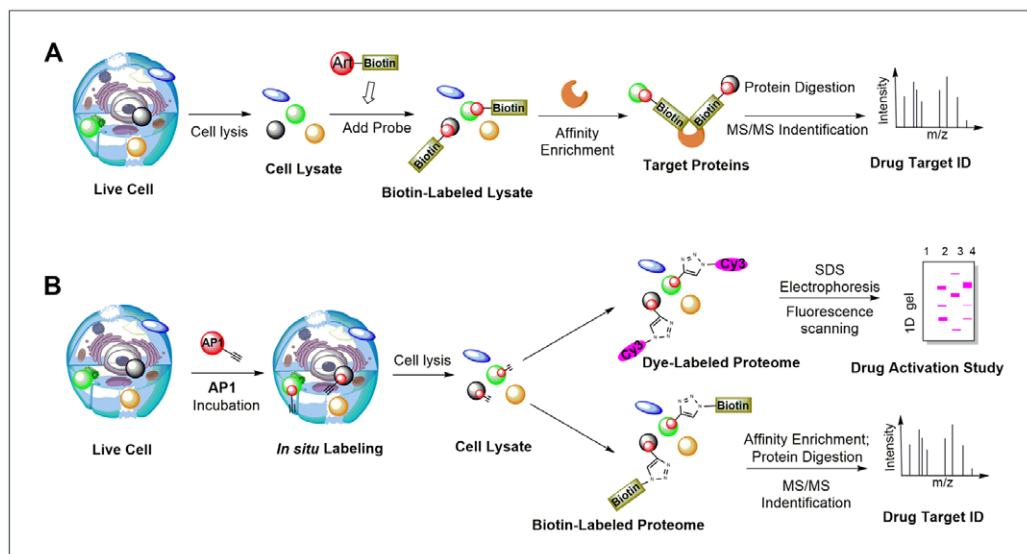


FIGURE 2 General workflow for chemical proteomics experiments referenced in this review. (A) Biotin-linked artemisinin probes such as BDHA (9) are added to cell lysates to label the proteome. Artemisinin-interacting proteins are then enriched by avidin, digested and analyzed by mass spectrometry (MS) to identify target proteins. (B) Click-chemistry functionalized probes such as the alkyne-linked AP1 (6) are incubated with live cells for direct in situ labeling. Treated cells are subsequently lysed and labeled via click chemistry, either with fluorescent tags for visualization in SDS gels or with biotin tags for enrichment and MS-based protein identification

protein expression studies using MALDI-TOF (matrix-assisted laser desorption/ionization–time-of-flight) MS and reported changes in the expression of ER stress and mitochondrial proteins following artemisinin treatment, while also characterizing differentially expressed proteins between artemisinin-susceptible and artemisinin-resistant HCT116 cells.^{55–57} In their follow-up study, a similar methodology with a more sensitive LC-MS/MS approach using resistant HeLa cells was applied, identifying a novel cytoprotective protein DJ-1 (encoded by the PARK7 gene) to be significantly and consistently overexpressed in artemisinin resistant cells.⁵⁸ DJ-1 was subsequently shown to be protective against oxidative damage via mitochondrial translocation and ROS removal, supporting the notion that ROS and mitochondria play important roles artemisinin-induced cell death. Xu et al. likewise applied LC-MS/MS combined with quantification by iTRAQ (isobaric tags for relative and absolute quantitation) to analyze gene expression changes in DHA-treated PC3 prostate cancer cells, highlighting the cellular protein synthesis machinery, amino acid metabolism, as well as the chaperone heat-shock protein, HSP70, as potential mediators of DHA-induced cytotoxicity in PC3 cells.⁵⁹ Considering that DJ-1 was just one of 30 identified proteins of interest in the Zhu's study and that the Xu's study identified up to 86 proteins, it is easy to appreciate that a high-sensitivity discovery-based platform such as MS-based proteomics can be invaluable in the identification of novel mediators of artemisinin activity.

To complement MS-based protein expression studies, the emerging field of quantitative chemical proteomics provides an avenue for a direct and activity-based profiling of drug–protein interactions. Chemical proteomics is based on the use of chemical probes that interact with their protein targets while carrying some form of reporter tag for visualization or purification.⁶⁰ These reporter tags include fluorescence groups for in-gel or even live cell visualization of interacting proteins, as well as affinity tags such as biotin that can allow for the specific purification of interacting proteins. These methods can be combined with quantitative methods such as iTRAQ and SILAC (stable isotope labeling with amino acids in cell culture) to provide quantitative information with regards to binding activity and protein abundance, thereby allowing an unbiased and comprehensive screen of interacting proteins^{60–62} (Fig. 2). In the context of artemisinin, this approach carries several critical advantages. First, as we have discussed in the previous section, chemical proteomics methods provide a direct method of visualizing drug activation as artemisinin needs

to be activated before exhibiting significant alkylating properties. Second, the direct profiling of interacting proteins can provide mechanistic information beyond that of gene expression studies.⁶³ Ravindra et al. first employed such an approach in their 2015 study of artesunate in an anti-asthmatic capacity, designing a biotin-modified probe of DHA BDHA (9) to purify and identify artesunate-interacting proteins in human bronchial epithelial cells⁶⁴ (Fig. 2A). This led to the identification of over 60 interacting proteins, with pathway analysis highlighting the glycolytic pathway and the mitochondrial proteins as potential mediators of the anti-inflammatory function of artesunate. Likewise in 2015, we developed an alkyne-linked artemisinin AP1 (6) to perform similar studies in *Plasmodium falciparum* to examine drug activation and targeting,⁴⁰ identifying a highly promiscuous mode of action for artemisinin in malaria, which was supported by a separate study using ABPP (activity based protein profiling) probes (10, 11) in 2016⁶⁵ (Fig. 2B). This method has subsequently been applied in the context of cancer, with results likewise supporting the promiscuous nature of artemisinin action in cancer.^{41,42}

Furthermore, MS-based proteomics enables the screening and identification of protein modification sites, including that of artemisinin-induced alkylation.⁶⁶ Xiao's group successfully made use of activity-based artemisinin probes to identify the binding sites of artemisinin in the well-known malarial target TCTP (translationally controlled tumor protein), establishing a methodology for identifying modification sites of key proteins of interest, which can provide relevant structural and functional information.⁶⁷ Strikingly, this study highlighted a highly nonspecific pattern of protein alkylation, where the artemisinin probe modified multiple adjacent residues on the analyzed fragment including phenylalanine, asparagine, threonine, and aspartic acid, in addition to the expected cysteine residues. The group proposes this to possibly be indicative of a random, proximity-based mechanism of targeting by the highly reactive heme-activated artemisinin. This could explain to an extent the apparent overlap between the proteins identified as artemisinin targets in parasite-based proteomics studies,^{40,63} and proteins previously identified as high-abundance proteins in parasitic proteomics analyses including the HSPs, metabolic proteins, and members of the protein synthesis pathway.^{68–70} Under this model, the efficacy of artemisinin may be considered to be primarily dependent on the extent of activation, while the downstream targets may be less specific and depend on factors including drug localization and proteome composition. Highly abundant proteins such as housekeeping proteins, as well as proteins proximal to artemisinin localization sites are thus more likely to be targeted, and changes to drug localization (e.g., by targeting the drug to specific organelles) may significantly alter its effects.⁴¹ The possibility remains that the intrinsic properties of certain proteins can render them more or less susceptible to artemisinin targeting—identifying such properties and understanding how different proteins might be affected under different cellular conditions will be crucial in advancing knowledge of the drug.

Finally, given the promiscuous nature and evident multifunctionality of artemisinin, it is important to note that the unbiased proteomics approach can easily be extended to other domains outside of cancer. A recent publication by Li et al. for instance details the use of affinity purification by immobilized artesunate and MS to identify the protein gephyrin as a mammalian target of artemisinin, thereby discovering a novel and promising link between artemisinin and pancreatic β cell regeneration in type 1 diabetes.⁷¹ Moving forward, it will be useful to consider the potential cross-talk between such noncancer mechanisms and cancer-related ones.

4 | ARTEMISININ-INDUCED CELL DEATH AND RELATED MECHANISMS

The general understanding of artemisinin-mediated cytotoxicity in cancer revolves around the induction of caspase-dependent, mitochondrial pathway mediated apoptosis. This is understood to be an outcome of ROS production following drug activation, leading to various downstream effects including oxidative stress, ER stress, and DNA damage that culminate in apoptotic cell death.²⁰ Notably, the availability of free cellular iron is known to play a major role in artemisinin-induced cell death. The chelation of free iron has been frequently reported to abrogate cell death, and the coadministration of artemisinin with transferrin and holotransferrin have been shown to potentiate its cytotoxicity in cancer.²⁰ These effects however have not been consistent among tested cell types, and nonapoptotic mechanisms for cell death have been reported.^{72,73} Growing evidence for a heme-centric activation mechanism for artemisinin has also

prompted a reexamination of the role of free iron in artemisinin-induced cell death. A robust understanding of the role of iron is significant for therapy design and the determination of artemisinin susceptibility for individual cancers. Here, we highlight novel findings related to the mechanism of cancer cell death following artemisinin treatment, focusing on nonapoptotic pathways including ferroptosis and autophagy as well as the role of iron in each of these processes.

4.1 | Apoptosis

The primary mechanism of artemisinin-induced cancer cell death is currently understood to be apoptotic cell death, which has been supported in a large number of publications following the first such report in 1996.⁷⁴ Artemisinin is frequently reported to induce apoptosis by the intrinsic mitochondrial pathway, mediated by caspase 3/9 activation and the release of cytochrome C following the permeabilization of the mitochondrial membrane.^{75,76} This form of artemisinin-induced cell death can be affected by the manipulation of antiapoptotic proteins such as Bcl-2, as well as proapoptotic factors such as Bid and Bak.^{77,78} Outside of the intrinsic pathway, the extrinsic/death receptor driven pathway has also been implicated in the action of artemisinin.^{79,80} As the apoptotic effects of artemisinin have been well-covered in recent reviews,^{14,18,20} we will instead focus on the nonapoptotic mechanisms of artemisinin-induced cell death in the rest of this section.

4.2 | Ferroptosis—iron-dependent cell death

A notable and emerging breakthrough in artemisinin-related cell death is the discovery of ferroptosis, a form of iron-dependent, nonapoptotic cell death.⁸¹ Ferroptosis is described to be morphologically, biochemically, and genetically distinct from other known forms of cell death. Most importantly, free iron chelation abrogates ferroptosis induction, but does not affect other forms of cell death such as apoptosis, while common inhibitors of apoptosis such as z-VAD-FMK fail to rescue cell death by ferroptosis.^{82,83} This is clearly a good fit for the iron-dependent nature of artemisinin-induced cytotoxicity and it is unsurprising that studies have emerged linking artemisinin to ferroptosis. Eling et al. first reported the role of artesunate as a specific inducer of ferroptotic cell death in pancreatic cancer (PDAC) cell lines.⁸⁴ Artesunate-induced cell death was determined to be iron- and ROS-dependent, and cell death was shown to be nonapoptotic and nonnecroptotic in nature. Cell death was fully abrogated by the selective ferroptosis inhibitor ferrostatin-1 (Fer-1), which suppresses lipid peroxidation but does not prevent apoptosis. Constitutively active mutant KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) correlated positively with artesunate susceptibility, but did not affect the protective effect of Fer-1. As ferroptosis regulation is genetically distinct from that of apoptosis, ferroptosis induction is a promising alternative for cancers carrying apoptosis-resistant mutations such as mutant KRAS in PDAC. Importantly, the authors raise the issue of identifying such genetic markers as a predictor of susceptibility to apoptotic and ferroptotic cell death. Expanding on this, Ooko et al. identified 20 genes in which mRNA expression correlated significantly with artemisinin efficacy in a panel of 60 NCI cell lines.⁸⁵ Among the 20, notable genes related to iron uptake and metabolism including transferrin, TfRs, and lactoferrin were identified, and ferroptotic cell death induction was again shown to be abrogated by both Fer-1 and DFO. Finally, Lin et al. reported on the effects of artemisinin in head and neck cancer cell line models, indicating the induction of both ferroptotic and apoptotic cell death.⁸⁶ DFO prevented both forms of cell death, confirming that iron remains strongly related to the artemisinin-related cell death by the canonical apoptotic pathway in certain cancer cell types. Looking forward, it will be important to verify the role of ferroptosis in artemisinin response in future studies, as well as acknowledge the possibility that different types of cell death may predominate in different cancer types.

4.3 | Autophagy and the lysosomal pathway

Autophagy is a highly conserved cellular degradation pathway in which cytoplasmic components, including proteins and organelles, are degraded and recycled. Portions of the cytoplasm are engulfed in double-membrane structures

known as autophagosomes and subsequently delivered to lysosomes, where the cargo is broken down by lysosomal enzymes.⁸⁷ While autophagy is mainly considered to be a prosurvival mechanism in response to nutrient deprivation, it is increasingly understood that the process is highly regulated and involved in many aspects of homeostasis and pathogenesis.^{88,89} In cancer, autophagy exerts both prosurvival and tumor-suppressive functions depending on the cellular context and the modulation of autophagy for cancer therapy is an area of increasing interest.⁹⁰ The connection between artemisinin and autophagy is thus important to ascertain. The manner in which autophagy contributes to cell death following artemisinin exposure and the possibility of modulating autophagy to enhance drug effectiveness are topics of particular interest.

One emerging link between autophagy and artemisinin-induced cytotoxicity is the mechanism of ferroptosis, the iron-dependent nonapoptotic cell death process described earlier. It has been reported that the process of ferroptosis requires a working autophagic machinery.⁹¹ Specifically, the lysosome-dependent degradation of cellular ferritin (intracellular iron storage proteins) has been shown to be necessary for ferroptosis.^{92,93} This autophagic degradation of ferritin, or ferritinophagy, may be an important source of cellular free iron that is in turn required for ferroptosis as well as the canonical iron-dependent generation of ROS.⁹¹ The role of the lysosome for artemisinin treatments was first reported by Hamacher-Brady et al., determining that lysosomal iron is necessary for artemisinin-induced ROS generation and that iron degradation in the lysosome occurs upstream of mitochondrial outer membrane permeabilization (MOMP) and eventual cell death.⁷⁶ We furthered explored the mechanism of artemisinin-lysosome interactions in our 2014 study, with the results firmly supporting a central role of the lysosome and autophagy in the response to artemisinin.⁹⁴ Using artesunate and HeLa cells, we found lysosomal localization and accumulation of artesunate as well as a corresponding activation of lysosomal functions, measured by an increase in lysosomal acidification and cathepsin enzyme activity. This was linked to an artesunate-mediated promotion of lysosomal V-ATPase assembly, with both the V-ATPase inhibitor bafilomycin A1 (BAF) and the lysosomal inhibitor chloroquine (CQ) significantly attenuating artesunate-induced cell death. Notably, suppression of ferritinophagy by NCOA4 (ferritinophagy adaptor) knockdown also exerted a protective effect against artesunate, further highlighting the importance of ferritin degradation. The induction of autophagy and lysosomal activity by artemisinin is thus a possible contributing mechanism to its effects, increasing the availability of cellular free iron to mediate ROS generation as well as Ferroptosis.

On the other hand, autophagy is known to have a cytoprotective role against oxidative stress, which occurs downstream of autophagy-mediated iron release.⁹⁵ There is thus a possible paradoxical effect where autophagy positively contributes to cell death by increasing iron availability, but protects against cell death by counteracting the effects of oxidative stress. It would be important in that case to identify the various determinants of the net result on artemisinin efficacy. Conflicting reports exist on the interplay between autophagy modulation and artemisinin response. As mentioned earlier, we found autophagy inhibition by both BAF and CQ (cotreatment with artesunate) to be protective against artesunate in HeLa.⁹⁴ In contrast, CQ pretreatment was shown to synergistically enhance artemisinin-induced cell death in nonsmall cell lung cancer (NSCLC) A549 cells, suggesting that the timing of autophagy inhibition could affect the cellular context and response to artemisinin.⁹⁶ The protective effect of autophagy following artemisinin treatment was shown more clearly by Chen et al., who identified the stress-related protein p8 to be upregulated following artemisinin exposure in HeLa and HCT116.⁹⁷ The protein p8, in combination with ER stress-related proteins ATF4 and CHOP, was shown to contribute to artemisinin-induced autophagy. Overexpression of p8 was protective against artemisinin, while p8 knockdown significantly sensitized cancer cells to apoptosis. Importantly, inhibition of autophagy by 3-MA or CQ pretreatment as well as Atg5 knockdown all served to increase apoptosis in artemisinin-treated cells and the combination of CQ and DHA was synergistic in its anticancer efficacy both *in vitro* and *in vivo*. Intriguingly, Button et al. reported not only the same enhancement effect by CQ on artemisinin-induced cytotoxicity, but also an inhibitory effect of artemisinin on autophagy in primary schwannoma cells.⁷² The nature of cell death in this case was further reported to be the necrosis-like programmed cell death mechanism known as necroptosis, rather than apoptosis. This could indicate the existence of cell-specific factors that could influence the interaction between artemisinin and autophagy, even though significant evidence exists for the proautophagic effect of artemisinin. It is clear that much

remains to be understood on the interplay between artemisinin, autophagy, and the various types of programmed cell death and cellular outcomes following artemisinin treatment.

5 | MOLECULAR TARGETS AND SIGNALING PATHWAYS IMPLICATED IN THE ANTICANCER POTENTIAL OF ARTEMISININ

Considering the promiscuous nature of artemisinin, it is not surprising that a wide range of signaling pathways and putative targets have been implicated in the anticancer mechanism of artemisinin. Following our discussions of artemisinin activation and artemisinin-induced cell death, we next highlight several novel functions and pathways from recent works that have yet to be reviewed. These include cellular functions such as cancer immunomodulation, metabolism, and stem cell activity as well as the STAT3 (signal transducer and activator of transcription 3) signaling pathway, and may offer new perspectives on the full scope of the mechanism of action of artemisinin.

5.1 | Immunomodulation in cancer

The role of artemisinin as an immunomodulator has drawn some attention in the recent years, with evidence of effects on both innate and adaptive immunity.^{12,98} Reports of positive effects on a range of autoimmune diseases and immune disorders have pointed to artemisinin as a promising immunoregulatory drug. In particular, artemisinin has been shown to exhibit immunosuppressive properties via the inhibition of T-cell activation and proliferation, as well as the ability to modulate regulatory T-cell (Treg) activity and cytokine production. Immune evasion and immunosuppression are among the hallmarks of cancer pathogenesis, where proliferating tumors prevent destruction by the immune system through CD4⁺CD25⁺FOXP3⁺ Treg recruitment and the production of immunosuppressive cytokines such as the interleukins IL-6 and IL-10 as well as TGF- β (transforming growth factor β).⁹⁹ Work by Noori and Farsam et al. first demonstrated the ability of artemisinin derivatives to deplete splenic Treg in mouse models with accompanying changes in IFN- γ and IL-4 response, although the evidence of IL-4 change is conflicting in the two published reports, reportedly due to chemical differences between the choice of artemisinin derivative used.^{100,101} The general pattern of Treg depletion following artemisinin exposure was supported by Zhang et al. in their 2014 study that reported a similar depletion of CD4⁺CD25⁺FOXP3⁺ Treg in mouse cervical cancer models, with a corresponding inhibition of PGE₂ (prostaglandin E2) production and FOXP3 downregulation.¹⁰² This reduction in PGE₂ corroborates with a subsequent study in smooth muscle cells.¹⁰³ Changes in signaling molecule production however is likely to be cell-line dependent, as demonstrated by Cui et al. in a later study that provided conflicting results with regards to the direction of change in PGE₂ production between two colorectal cancer cell lines (Colon26 and RKO), although both tested cell lines displayed significant reductions in TGF- β production.¹⁰⁴ As a point of contrast, artemisinin has also been reported to significantly promote Treg generation through mTOR (mammalian target of rapamycin) modulation in noncancer conditions, underscoring the need to further clarify the application of artemisinin and its immunomodulatory properties in the context of cancer.¹⁰⁵ The anti-immunosuppressive effects of artemisinin could well be a contributing factor in its efficacy in combinatorial treatments and the potential role of artemisinin in cancer immunotherapy is an exciting prospect.

5.2 | STAT3

STAT3 is a transcription factor involved in cell survival and proliferation, cell cycle processes, as well as immune responses.^{106,107} Constitutively active STAT3 is associated with the induction and proliferation of many forms of cancer, and often signals a poor prognosis.^{108–111} Targeting STAT3 is thus a novel and promising approach in cancer therapy, and the development of STAT3 inhibitors is an area of considerable interest.¹¹² However, direct targeting of STAT3 at a pharmacologically relevant level has proved to be difficult and no clinical trials are currently in place

for direct STAT3 inhibitors in cancer therapy.¹⁰⁷ As a cancer-specific promiscuous agent, artemisinin has recently been reported to be a potential direct inhibitor of STAT3. In their 2016 publication, Jia et al. carried out a study in head and neck squamous cell carcinomas (HNSCC) using DHA in both *in vitro* and *in vivo* models, demonstrating selective inhibition of STAT3 activity in constitutively active STAT3 cell lines as well as xenograph tumors at a level comparable to the experimental inhibitors AZD1480 and AG490.¹¹³ This was shown to be a result of Jak2 phosphorylation blockade, and came with the expected inhibition of HNSCC proliferation both *in vitro* and *in vivo*. This effectiveness against STAT3 was supported by Ilamathi et al. in their investigation of sesquiterpene lactone compounds in hepatocellular carcinoma (HCC), where artesunate was identified to be the most effective inhibitor of STAT3 with comparable potency to the experimental inhibitor S31-201.¹¹⁴ Rather than Jak-mediated activity however, Ilamathi et al. reported a direct effect on STAT3 dimerization and DNA binding, possibly acting through the SH2 domain. Outside of cancer, inhibition of STAT3 phosphorylation and activation following artemisinin or artemisinin analogue exposure have also been reported in the context of adipocytes and rheumatoid arthritis, for the application of artemisinin in obesity and autoimmune diseases, respectively.^{115,116} It will be interesting to explore the possibility of applying artemisinin in this role given its proven safety and clinical efficacy compared to other STAT3 inhibitors.

5.3 | Cancer metabolism

It is well-known that cancer cells exhibit altered metabolic patterns compared to their healthy counterparts. These changes can be reflected in a general elevation of metabolic rates to support increased proliferation, as well as a shift in preferred energy source from oxidative phosphorylation to glycolysis under the so-called “Warburg effect.”¹¹⁷ Targeting the metabolic differences between cancer and normal cells thus holds some promise as an anticancer strategy. The role of artemisinin in this regard has remained relatively unexplored until the recent years, especially in the context of cancer. Mi et al. first reported a series of glucose metabolism-related effects of artemisinin in NSCLC cell lines.¹¹⁸ These effects included an inhibition of glucose uptake and a corresponding attenuation of glycolytic metabolism in the form of suppressed ATP and lactate production. These were linked to a suppression of mTOR activity as well as a reduction in the expression of the glucose transporter GLUT1. Notably, artemisinin synergized with the glycolysis inhibitor 2DG (2-deoxy-D-glucose) to inhibit proliferation and induce apoptosis in NSCLC cells, indicating a potential use of artemisinin as a cancer-specific modulator of glucose metabolism. Other more direct effects of artemisinin on the glycolytic machinery were suggested by several independent proteomics studies. An untargeted chemical proteomics screening by Ravindra et al. in human bronchial epithelial cells identified eight enzymes in the glycolytic pathway (out of 11 constituent enzymes) as artesunate-interacting proteins, suggesting a possible effect of the drug on glucose metabolism.⁶⁴ This observation was replicated in cancer cells independently by Zhou et al. and our laboratory in chemical proteomics screenings, with all 11 glycolytic pathway-related enzymes being identified as artemisinin targets in our data set.^{41,42} Aside from glycolysis, other implicated metabolic pathways including amino acid biosynthesis and fatty acid synthesis have also been reported in these data sets, as well as in other noncancer studies.¹¹⁹ Given the promiscuous nature of artemisinin targeting, these effects on cellular metabolism may not be a surprising outcome. Nevertheless, the metabolic influence of artemisinin may constitute a significant aspect of the drug’s mechanism.

5.4 | Cancer stem cells

The cancer stem cell (CSC) model describes a subtype of cancer cells with stem cell like characteristics that are responsible for driving tumor initiation, metastasis, resistance to treatment, and relapse.¹²⁰ The selective targeting and suppression of such CSCs is thus an area of great interest for cancer research.¹²¹ Several recent publications have indicated an antistem cell aspect of artemisinin. Cao et al. first performed such a study in glioma stem cell (GSC) culture systems, reporting proapoptotic effects similar to the well-described anticancer properties of artemisinin in nonstem cancer cell populations.¹²² The effects of artemisinin on stem cell populations in cancer were further

described in two other studies. Tong et al. described a suppressive effect on NSCLC CSCs by artemisinin, as reflected by a reduction in CSC markers such as Sox2 and Oct4.¹²³ This was linked to an artemisinin-mediated inactivation of the Wnt/ β -catenin pathway, which is a well-documented mechanism of action for artemisinin in the context of cancer. Separately, Berte et al. investigated the combination of artemisinin and temozolomide (first-line chemotherapy drug for glioblastoma) in glioma and glioma stem like cell lines.¹²⁴ Artemisinin alone or in combination with temozolomide reduced cell viability, and artemisinin enhanced the killing effect of temozolomide in both glioma and glioma stem-like cells. Artemisinin-mediated inhibition of temozolomide-induced senescence as well as DNA homologous recombination were suggested as possible mechanisms for this effect. Notably, artemisinin was also solely identified in a high-throughput screening study by Subedi et al. of approximately 6000 compounds as a selective inhibitor of cancer stemness.¹²⁵ Using a model system of induced cancer stem like (iCSCL), the artemisinin analogue NPD2604 as well as artemisinin and artesunate were found to selectively and potently inhibit CSCs as determined by alkaline phosphatase assays and cell viability screenings between monolayer and 3D tumor sphere cultures. This inhibition was shown to be independent of free iron, but dependent on mitochondrial metabolism and artesunate-induced mitochondrial dysfunction. Further study will be required to ascertain the role of artemisinin as a possible inhibitor of CSCs in addition to its antineoplastic properties. Table I summarizes the studies discussed in this section.

6 | POTENTIAL APPLICATION IN CANCER THERAPY

Artemisinin and its derivatives are promising prospects in cancer therapy for a few key reasons. Apart from cancer selectivity and its status as an established and relatively safe drug, the fact that it targets multiple hallmarks of cancer through a range of pathways and induces cell death through multiple mechanisms makes it an attractive option for a heterogeneous disease such as cancer. With artemisinin being relatively inefficacious as a monotherapy, it is important to consider methods of enhancing its effects in order to design clinically relevant treatments. Here, we will discuss relevant findings in drug resistance, as well as drug synergy and combination therapy. Novel approaches to enhance the efficacy of artemisinin will also be briefly outlined.

6.1 | Artemisinin resistance

Reports of artemisinin resistance in cancer is relatively limited compared to the volume of research detailing its efficacy. Nevertheless, knowing the mechanisms and identifying the predictive markers of drug resistance is essential for a complete understanding of the drug mechanism as well as its applicability in therapy. The possibility of drug resistance to artemisinin was first reported in 2011 by Bachmeier et al., highlighting the induction of resistance in the highly metastatic MDA-MB-231 breast cancer cell line.¹²⁶ Following artesunate pretreatment, MDA-MB-231 cells exhibited a reduction in response over 24 hr of treatment compared to less invasive MDA-MB-468 cells. This effect was linked to an activation of the NF- κ B and AP-1 (activator protein 1) transcription factors, a corresponding increase in MMP-1 (matrix metalloproteinase 1) expression as well as the prevention of apoptosis. Working models of artemisinin resistance in cancer were subsequently established by independent groups, using continued and gradually increasing exposure to generate artemisinin-resistant HCT116 human colon cancer and Molt-4 human lymphoblastoid cells.^{56,127} In the HCT116 study, artemisinin resistance was not linked to common multidrug resistance (MDR) markers such as MRP1 (multidrug resistance protein 1) upregulation, or no obvious difference in drug efflux was observed using doxorubicin as a control. Proteomics analysis identified eight differentially expressed proteins between sensitive and resistant cells, including components of the glycolytic pathway as well as the stress response/chaperone proteins HSP27 and HSP90.⁵⁶ On the other hand, the resistant Molt-4 model demonstrated resilience against artemisinin-induced DNA damage, but interestingly was not resistant to artemisinin-tagged holotransferrin (ART-TF).^{127,128} This raises questions about the relationship between drug uptake and artemisinin efficacy, specifically with regards to the role of transferrin and its receptor. Indeed, artemisinin intake has been reported to depend on TfR1 function.^{73,129} It would

TABLE I Summary of Pathway and Target Identification Studies Discussed in Section 3

Study	Findings	Methodology
Cancer immunomodulation		
Noori and Hassan (2011) ¹⁰⁰	IL-4↓, CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Treg↓	DHA treatment in mammary tumor-bearing Balb/c mice
Farsam et al. (2011) ¹⁰¹	IL-4↑, CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Treg↓	Artemether treatment in mammary tumor-bearing Balb/c mice
Zhang et al. (2014) ¹⁰²	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Treg↓, PGE ₂ ↓, FOXP3↓	Artesunate treatment in U14 cervical cancer-implanted mice and cervical cancer cell lines
Cui et al. (2015) ¹⁰⁴	TGF-β1 and IL-10↓, immunosuppression inhibited	Artesunate treatment in colorectal cancer Colon26 and RKO cell lines
STAT3		
Jia et al. (2016) ¹¹³	Jak2/STAT3 signaling inhibited	DHA treatment in HNSCC cell lines
Ilamathi et al. (2016) ¹¹⁴	Artesunate identified as potential STAT3 inhibitor, interferes with IL-6 driven STAT3-DNA binding, inhibits STAT3 dimerization	Screen of sesquiterpene lactone compounds in hepatocellular carcinoma cell lines
Cancer metabolism		
Mi et al. (2015) ¹¹⁸	Glycolysis↓, mTOR activation↓, GLUT1↓	DHA treatment in NSCLC cell lines
Ravindra et al. (2015) ⁶⁴	8 Glycolytic enzymes identified as ART-binding proteins	Chemical proteomics screening with artemisinin probe in human endothelial cells
Zhang et al. (2016) ⁴¹	11 Glycolytic enzymes identified as ART-binding proteins; amino acid and fatty acid synthesis identified as possible implicated pathways	Chemical proteomics screening with artemisinin probe in live HCT116 cells
Cancer stem cells		
Cao et al. (2014) ¹²²	Induction of cell cycle arrest and apoptosis in glioma stem cell populations	DHA treatment in enriched glioma stem cells
Tong et al. (2016) ¹²³	Cancer stem cell markers SOX2, OCT4, NANOG↓	Artemisinin, artesunate, DHA treatment in NSCLC cell lines
Berte et al. (2016) ¹²⁴	Killing effect of temozolomide in glioma stem-like cells enhanced by artesunate treatment	Artesunate treatment in glioblastoma cell lines and glioblastoma stem-like cells
Subedi et al. (2016) ¹²⁵	Artesunate identified as selective inhibitor of induced cancer stem-like cells through the induction of mitochondrial dysfunction	High-throughput screening of over 6000 compounds; artesunate treatment of induced cancer stem-like cells
Other pathways and functions		
Kim et al. (2015) ¹⁷⁹	p38, ERK, CREB, Chk-2, STAT5, RSK phosphorylation↓, SOCS-1 expression↑	Artesunate treatment in human CML cell lines and nude mice xenografts
Chen et al. (2015) ¹⁸⁰	Disintegrin and metalloproteinase 17 (ADAM17) expression↓	DHA treatment in glioma cell lines
Target profiling		
Zhang et al. (2016) ⁴¹	321 Proteins identified as artemisinin-binding proteins	Chemical proteomics screening with artemisinin probe in live HCT116 cells
Zhou et al. (2016) ^{41,42}	79 Proteins identified as artemisinin-binding proteins	Chemical proteomics screening with artemisinin probe in HeLa lysate

thus appear that drug influx as well as intrinsic cellular resistance toward oxidative stress, DNA damage, or protein alkylation could all be relevant in determining artemisinin resistance, especially considering the promiscuous nature of artemisinin. Drawing from malaria parasite research, the prominent resistance-related mutation in kelch13 has in fact been linked by Dogovski et al. to the ubiquitin-proteasome system, in a model where artemisinin resistance in parasites can be linked to the enhanced clearance of damaged proteins and a more robust stress response.¹³⁰ The coadministration of proteasomal inhibitors and DHA synergistically enhanced the effectiveness of DHA both *in vitro* and *in vivo*, a result that remains to be validated in cancer models. Finally, other possible mechanisms of resistance including drug efflux mechanisms as well as the regulation of artemisinin activation remain relatively unexplored and would likewise be interesting directions for future study.

6.2 | Enhancement of anticancer activity

Pharmacologically, artemisinin and its derivatives are extremely effective in their antimalarial role, but less potent as anticancer drugs (especially as monotherapy) due to less than ideal pharmacokinetic properties.¹³¹ The elimination half-life of DHA in humans lies between 1 and 2 hr, necessitating daily doses and combination with longer acting antimalarials for maximum efficacy.¹³²⁻¹³⁴ Such limitations are likely to be significantly accentuated in cancer treatment, which may necessitate far longer treatment courses and raise concerns of toxicity as well as compliance. While the favorable properties and advantages of artemisinin ensure its relevance as a promising development target, it is clear that strategies must be devised to enhance its potency. Apart from combination therapy which will be discussed later, a variety of methods have been explored ranging from novel delivery methods and new formulations to specific organelle targeting. Drug encapsulation methods, especially at the nanoscale with liposomes and niosomes have also been drawing interest as a promising approach in nanomedicine. In principle, drug encapsulation can improve specificity and bioavailability as well as alter pharmacokinetic properties of the drug.¹³⁵ The recent years have seen a remarkable surge of studies exploring various approaches in nanomedicine, ranging from liposomal delivery,¹³⁶⁻¹⁴¹ encapsulation with other drugs or biomolecules including transferrin,¹⁴²⁻¹⁴⁵ encapsulation with specific properties such as magnetic or pH-responsive liposomes,¹⁴³⁻¹⁴⁶ novel metal based nanostructures,^{146,147} solid lipid nanoparticles (SLNs), and niosomes.¹⁴⁸ In general, these studies have consistently reported positive results with significant enhancements to efficacy and improved selectivity. Several approaches have also been shown to offer additional advantages and new possibilities. For instance, magnetic liposomes could in theory be used for precise drug targeting, while the dual metal organic framework (dual-MOFs) approach taken by Wang et al. can serve as imaging tools in addition to being drug delivery mechanisms.^{143,146} While we cannot review the vast amount of literature available in this rapidly expanding field in detail here, it would definitely be prudent to refer to these advances in biomaterial and nanomedicine research when considering the future direction of artemisinin therapy.

The development of more efficacious and pharmacokinetically favorable analogues of artemisinin is perhaps the most intuitive solution to its current limitations. One such synthetic derivative, OZ439 (artefenomel), has been shown to be an effective and promising alternative to the artemisinins in the context of malaria.¹⁴⁹⁻¹⁵¹ By stabilizing the peroxide pharmacophore, OZ439 exhibited increased half-lives of over 20 hr in rat models and over 24 hr in humans. With enhanced pharmacokinetic properties and comparable antiparasitic efficacy to the artemisinins, OZ439 and other similar approaches could prove promising at least in an antimalarial setting, although cancer-focused investigations remain limited and preliminary.¹⁵⁰ In the same vein, novel formulations such as synthetic dimers, trimers, and drug hybrids with quinolines as well as other compounds have also emerged as viable methods of drastically enhancing drug effectiveness as well as overcoming drug resistance and improving pharmacokinetic properties.¹⁵²⁻¹⁶¹ Many of these synthetic derivatives have been extensively reviewed by the Tsogoeva group.¹⁶²

Another emerging approach focuses on targeted drug delivery to specific cellular compartments and organelles. In the context of artemisinin, the mitochondria are not only implicated in ROS-related activities and the triggering of mitochondrial apoptosis, but are also the site of cellular heme synthesis that may be relevant for drug activation. Several groups have attempted the mitochondrial approach with some success.^{41,163} To accomplish this, the mitochondrial targeting fluorophore coumarin-3-carboximide was used by X. Zhang et al. as a coumarin-artemisinin conjugate

(12), while C.-J. Zhang et al. made use of the lipophilic cation group TPP (triphenyl phosphonium) linked to artemisinin (7).^{164,165} In each case, mitochondrial localization was achieved and the mitochondria-targeted drug exhibited significantly higher cytotoxicity compared to the normal drug, reaching up to 94-fold in the C.-J. Zhang study. Continued development of such novel approaches will be key for the application of artemisinin in cancer treatment.

6.3 | Artemisinin in combinational therapy

With its diverse mechanisms of action, the potential of artemisinin for synergy and enhancement in combination therapy is of particular interest. Not only is artemisinin less likely to merely overlap in mechanism with other treatments, the multitarget nature of artemisinin means it could be less susceptible to chemoresistance.

Artemisinin cotreatment has been shown to synergize with or enhance the effects of various conventional chemotherapy drugs in a range of cancer types (Table II). These include DNA alkylating and intercalating agents including cisplatin,^{113,166,167} carboplatin,¹⁶⁸ doxorubicin,^{161,169,170} temozolomide,^{124,171,172} the antimetabolite cytarabine,^{131,161} as well as kinase inhibitors such as sorafenib and midostaurin.^{73,161,173} The application of the artemisinin–temozolomide combination in gliomas has been shown by multiple reports to be especially efficacious. These effects have been reported to be mediated by multiple mechanisms including autophagy induction and the inhibition of homologous recombination and temozolomide-induced senescence, and this combination could be well worth further study. The combination with sorafenib raises another point of particular interest, with sorafenib having recently been identified as a ferroptosis inducer much like artemisinin itself.⁸² Noteworthy synergy between sorafenib and artemisinin have been reported in renal cell carcinoma and leukemia cells *in vitro*, but the effect of this combination on ferroptosis as well as the role of ferroptosis inducers as a whole in cancer combination therapy remain relatively unexplored.¹⁷⁴ Finally, artemisinin has also been reported to sensitize cervical cancer cells to radiotherapy as well as enhance the effects of photodynamic therapy in esophageal cancer cells.^{175,176} Continued effort in the discovery and testing of effective and safe artemisinin combinations will be crucial in its repurposing into an anticancer role.

7 | SUMMARY AND OUTLOOKS

As a novel anticancer drug, artemisinin and its derivatives possess a host of advantages that make it worthy of development. Artemisinin is selective, efficacious against an extremely wide range of cancer types in cell and living models, and simultaneously address multiple hallmarks of cancer that makes it an effective agent in combination therapy as well as less susceptible to resistance. Perhaps most importantly, it is already an established drug for the treatment of malaria, with proven safety records in clinical use. Clinical trials of artemisinin as an anticancer drug, though limited in number, have been generally encouraging both in terms of efficacy as well as the degree of toleration in patients.^{14,20,179,180} While a good body of work already exists to paint a general picture of the drug's mechanism in cancer, several key gaps in knowledge remain and we have attempted to highlight the salient issues in this review (Fig. 3).

First, the nature of drug activation in cancer and the identity or identities of artemisinin activators remain to be elucidated. While free ferrous iron is frequently cited as the principal activator due to its undeniable involvement in downstream ROS generation and cytotoxicity, increasing evidence points to the role of heme as a possible principal activator instead. We propose that the role of iron could be considered as being necessary for downstream processes and heme synthesis rather than as the direct or primary activator of artemisinin. Further study will be required to unambiguously define the roles of heme and iron in terms of drug activation.

Second, cellular targets and protein mediators of cellular effects must be precisely identified. A multitude of pathways have been implicated in the effects of artemisinin, but few have been characterized in terms of the exact protein or proteins being modified by the drug. We have highlighted emerging high-throughput proteomics methods in identifying these targets, but it is also important to keep in mind that targets are likely to be dependent on cellular context and cancer type. The role of artemisinin in modulating cancer immunosuppression and cancer metabolism may be of particular interest moving forward.

TABLE II Summary of Artemisinin Combinations with Chemotherapy Drugs

Drug combination	Drug type	Cancer model	Involved pathway(s)	Reference
Carboplatin	Cross-linking agent	Ovarian cancer cell lines A2780, OVCAR-3	Activation of mitochondria-dependent, caspase-dependent apoptosis	Chen et al. (2009) ¹⁶⁸
Cisplatin	Crosslinking agent	Ovarian cancer cell line SKOV3	mTOR inhibition, activation of apoptosis	Feng et al. (2014) ¹⁶⁶
		Ovarian cancer cell line A2780, HO8910	RAD51 downregulation and impairment of DNA double-stranded break repair	Wang et al. (2015) ¹⁶⁷
		Head and neck cancer cell lines FaDu, Cal-27, Hep-2	STAT3 inhibition	Jia et al. (2016) ¹¹³
Cytarabine	Antimetabolite	Ten human AML (acute myeloid leukemia) cell lines and primary patient blasts	ROS generation, lysosomal disruption, induction of apoptosis	Drenberg et al. (2016) ¹³¹
Doxorubicin	Intercalating agent	Breast cancer cell line MCF-7	Activation of mitochondria-dependent, caspase-dependent apoptosis	Wu et al. (2013) ¹⁶⁹
		Cervical (HeLa), ovarian (OVCAR-3), breast (MCF-7), colorectal (PC-3), and lung (A549) cancer cell lines	Activation of mitochondria-dependent, caspase-dependent apoptosis	Tai et al. (2016) ¹⁷⁰
Temozolomide	Alkylating agent	Glioma cell lines U87MG, A172	Cell cycle arrest, induction of apoptosis and necrosis	Karpel-Massler et al. (2014) ¹⁷¹
		10 human glioma cell lines, nude mice	Autophagy induction	Zhang et al. (2015) ¹⁷²
		Glioma cell lines LN229, A172, and U87MG	ROS and necroptosis induction, RAD51 downregulation and inhibition of homologous recombination, inhibition of temozolomide-induced senescence	Berte et al. (2016) ¹²⁴
Sorafenib	Tyrosine kinase inhibitor, ferroptosis activator	Hepatocellular carcinoma cell lines HepG2 and BWTG3	Angiogenesis inhibition	Vandewynckel et al. (2014) ¹⁷³
		Renal cell carcinoma cell lines Caki-1, 786-O, and SN12C-GFP-SRLu2	G2/M cell cycle arrest	Jeong et al. (2015) ⁷³
Multiple antileukemic drugs (cytarabine, doxorubicin, etoposide) and kinase inhibitors (midostaurin, lestaurtinib, sorafenib)		Acute leukemia cell lines MOLM14 and KOPN8		Fox et al. (2016) ¹⁶¹

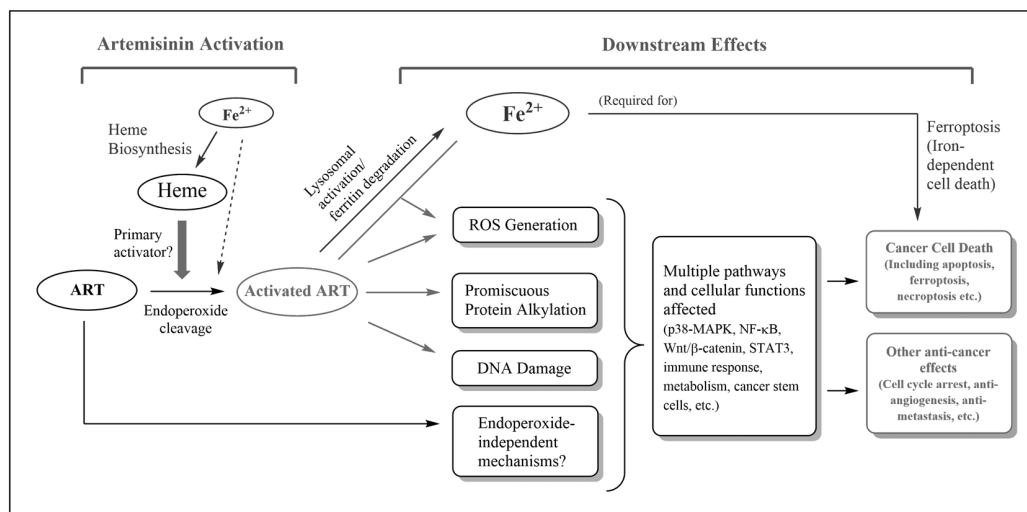


FIGURE 3 General scheme of the mechanism of action of artemisinin (ART) in cancer. Heme (which requires iron for its biosynthesis) serves as the primary activator of artemisinin, rather than free iron. The effect of iron manipulation (e.g., chelation by DFO) at this stage is thus indirect, and is mediated by the effect on heme availability rather than iron levels. Following activation, artemisinin effects changes in multiple signaling pathways through ROS generation, the promiscuous targeting of cellular proteins as well as DNA damage, culminating in cell death which can take both apoptotic and nonapoptotic forms, including the iron-dependent form of programmed cell death known as ferroptosis. Other downstream effects include the induction of cell cycle arrest, as well as the suppression of angiogenesis and metastasis, among others. While it is not directly responsible for artemisinin activation, iron remains intimately linked to the effects of artemisinin through its contribution to heme synthesis, ROS generation, and iron-dependent cell death. Artemisinin itself may also affect the pool of cellular iron through lysosomal activation and the stimulation of autophagic ferritin degradation

Third, the global understanding of the mechanism of action remains highly incomplete. It is increasingly clear that cytotoxicity is not only the result of iron-mediated ROS generation and apoptosis. We have described the role of the iron-dependent, nonapoptotic cell death termed ferroptosis, which is in turn dependent on the degradative function of autophagy and lysosomal function. Other iron-dependent as well as iron-independent mechanisms have also been proposed, painting a complex picture that seems to depend once again on cellular context and the type of cancer. Identifying the dominant or most important contributor to cell death in each cancer type and environment will be crucial in determining the effectiveness of artemisinin-based treatments.

Lastly, the repurposing of artemisinin from its antimalarial role to an anticancer one will require the overcoming of pharmacological limitations. Combination therapy and other strategies to enhance the effect and specificity while minimizing off-target effects will be important to consider. Much more will need to be done in terms of both mechanistic and clinical studies before repurposing is possible.

Looking ahead, it will be important to keep these issues in mind in the continuing pursuit of a novel and effective anticancer therapeutic strategy in artemisinin. With its promiscuous nature, it is likely that there will be novel benefits and mechanisms yet to be discovered, in diseases not limited to cancer. The existing evidence is more than sufficient to highlight the incredible potential of artemisinin, and the future of this Nobel-winning compound promises to hold many exciting possibilities.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

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